STUDIES IN THE CATABOLISM OF CHOLESTEROL BY MAMMALS

by

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CONTENTS

CONTENTS (Contd.)

SECTION I

INTRODUCTION

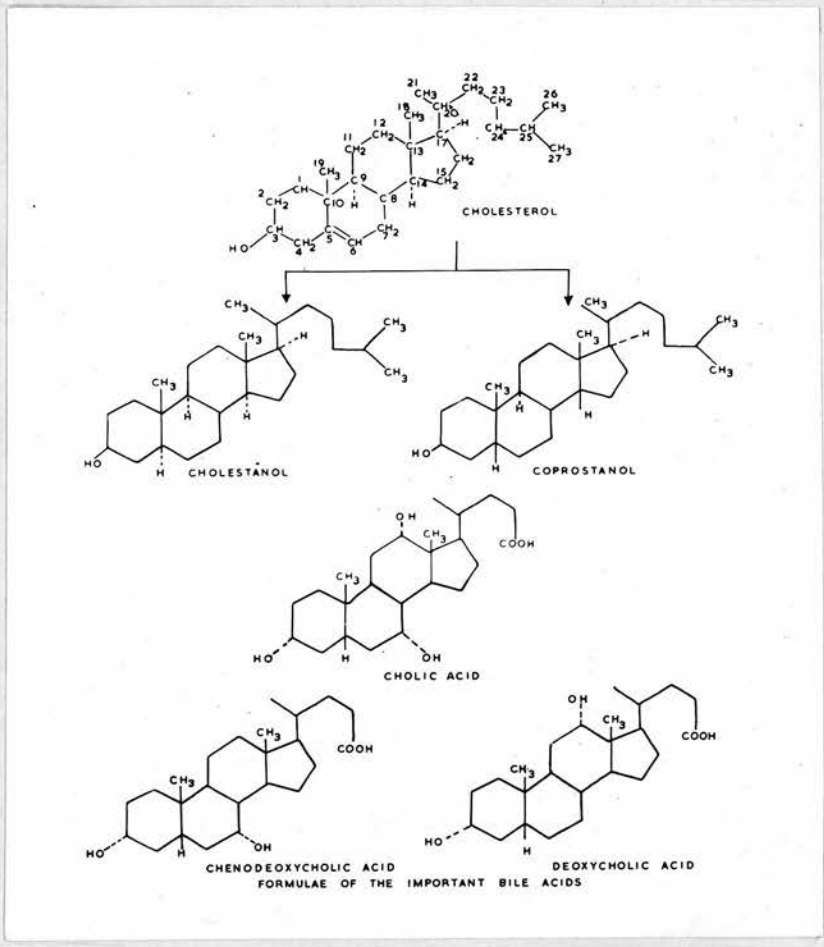
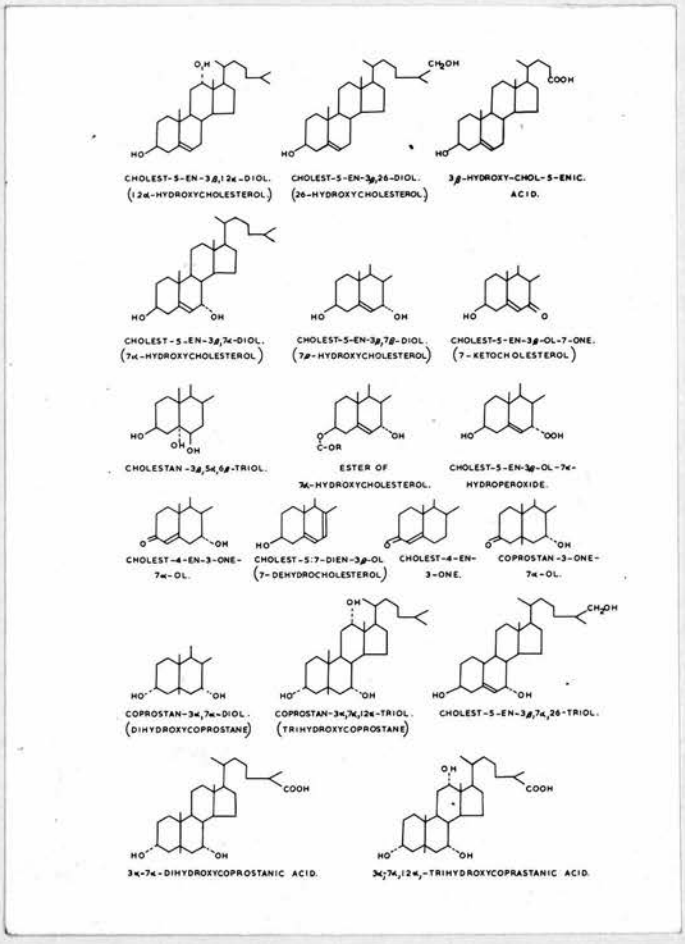

CHOLESTEROL
CHOLESTANOL
COPROSTANOL
CHOLIC ACID
CHENODEOXYCHOLIC ACID
DEOXYCHOLIC ACID
FORMULAE OF THE IMPORTANT BILE ACIDS

Figure 1



Formulae of steroids mentioned in the text

Figure 2

INTRODUCTION

The Chemistry of Sterols

The extraction of a plant or animal tissue with organic solvents yields an appreciable quantity of lipid material which is resistant to saponification. Such unsaponifiable lipids may include vitamins, ubiquinone and one or more of a variety of organic substances belonging to a group of crystalline alcohols known as sterols. In the tissues of vertebrates the principal sterol is the C_{27} alcohol, cholesterol. The classical work of Wieland, Windaus, Diels, Rosenheim and King which is reviewed by Fieser and Fieser (1959) led to the formulation of the structure of cholesterol, the fundamental carbon skeleton being the cyclopentanoperhydrophenanthrene ring, as shown in Fig 1. This consists of three six-membered rings fused together with one five-membered ring. At C_{17}, an iso-octane side chain is attached.

In this molecule there are several asymmetric centres, the angular methyl group at C_{10} being taken as the reference. Oneof these centres is at position 3, where the hydroxyl group can be either above or below the plane of the ring.

α-Substituents, which are below the plane of the ring and therefore trans to the methyl group at C_{10} are indicated by dotted lines, and β-substituents, which are above the plane of the ring and cis to the reference group are shown by solid lines. Thus, cholesterol has a hydroxyl group in the 3β-position. There is also a potential asymmetric centre at position 5. Thus, if in cholesterol the double bond between C_5 and C_6 becomes saturated, two series of compounds can arise. Compounds with the hydrogen in the α-position at C_5, i.e., trans to the reference group, belonging to the cholestane series, whereas the series of compounds which have the hydrogen in the β-position, cis to the reference group, is known as the coprostane series.

The alcohol, cholestanol, derived from cholestane, is given the systematic name of cholestan-3β-ol. In partially unsaturated sterols, such as cholesterol, the presence of a double bond is indicated by the suffix "ene". Thus, the systematic name for cholesterol is cholest-5-en-3β-ol. The sterochemistry and nomenclature of steroids are reviewed by Klyne (1957).

Occurrence of Cholesterol

In mammals cholesterol is present in all tissues, and it would seem, in all cells and parts of cells. It is found both free and esterified at the 3β-position with various fatty acids. For example, in liver, the predominant esterified fatty acid is linoleic acid, a C_{18} poly-unsaturated, methylene-interrupted fatty acid, and then oleic and palmitic acids. The short chain fatty acids are not found to any great extent.

In most animals the adrenal cortex is rich in cholesterol esters, whereas in nervous tissue, which also contains a large amount of sterol, it is found entirely in the free form. Tissues, such as liver, kidney and lungs, are intermediate in cholesterol content, whereas bone, muscular tissue and cartilage are low in sterol.

Cholesterol is found in all parts of cells. In liver, nuclei and cell debris contain 50% of the cell cholesterol, microsomes 25% and mitochondria and supernatant about 10% each (Kritchevsky, Langan and Whitehouse, 1960). Cholesterol is solubilised in the form of lipo-protein complexes, which appear to be an integral part of cell membranes.

Enzyme preparations have been obtained from liver, pancreas and plasma which will both esterify cholesterol with fatty acids, such as oleic acid, and also hydrolyse such esters (Sperry and Brand, 1941; Deykin and Goodman, 1962 (a), 1962 (b); Swell, Byron and Treadwell, 1950; Swell and Treadwell, 1950).

In general, the functions of cholesterol esterification are not well understood. It may be of significance that brain, with the lowest turnover rate of cholesterol of any tissue in the body, has essentially no cholesterol in the esterified form, whereas adrenal cortex, liver, and plasma with rapid turnover rates have relatively large fractions of cholesterol in the esterified form.

Auto-oxidation of Cholesterol

Cholesterol is readily oxidised in colloidal aqueous solution when exposed to excess oxygen. The chief products of this auto-oxidation are cholest-5-en-3β-ol-7-one (7-keto-cholesterol), cholest-5-en-3β-7β-diol and cholest-5-en-3β-7α-diol (7β- and 7α-hydroxycholesterol, respectively), and their dehydration products (Bergstrom and Wintersteiner, 1941, 1942). This shows that the

7-position, which is allylic, must be a very reactive one, and *in vitro* studies on the metabolism of cholesterol in tissue preparations are made very difficult by the formation of these products non-enzymically.

In liver cells, 99% of the sterol present is cholesterol and its esters, and only trace amounts of any other sterols have been found. Reports in the literature of the isolation of large amounts of these 7-oxygenated substances are open to question, as they are most likely artefacts formed during the actual extraction procedure.

Biosynthesis of Cholesterol

Although dietary cholesterol is readily absorbed by higher animals, it is not an essential component of their diet, and the fact that most animals can make their sterols from smaller carbon compounds has long been known.

The use of isotopic methods has provided important information about the metabolic pathways in sterol synthesis, and the studies of Bloch and co-workers (1954), and of Cornforth and associates (1953, 1957) have led to the elucidation of these pathways. Early experiments had shown that

acetic acid is an effective precursor of cholesterol. In fact, it was shown that all the 27 carbon atoms of cholesterol were derived from the carbon atoms of acetate. Very briefly, the steps in the formation of cholesterol from acetate have been shown to be:-

acetate → mevalonate → farnesenic acid → squalene, which is cyclised to give lanosterol → cholesterol

The details of cholesterol biosynthesis can be found in a recent review by Popjak and Cornforth (1960).

Although the liver is of major importance in cholesterol synthesis, all other animal tissues are capable of performing this process. As judged by isotope data, the half-life of cholesterol in the liver of the intact rat is about 6 days; in the extra-hepatic tissues the half-life is about 32 days.

Degradation of Cholesterol

Using isotopically-labelled cholesterol it has been shown that it can be metabolised to various classes of compounds in the tissues of the body. In the adrenal glands, progestins,

together with oestrogens and androgens are formed and the latter hormones in the reproductive glands.

Cholesterol may be metabolised to Vitamin D_3. Glover, Glover and Morton (1952) showed the reversible formation of cholest-5:7-diene-3β-ol (7-dehydrocholesterol) from cholesterol in the intestinal wall of the guinea-pig. 7-Dehydrocholesterol is converted in skin and *in vitro* into vitamin D_3, on irradiation with ultra-violet light. More recently, however, Mercer and Glover (1961) suggested that 7-dehydrocholesterol and lathosterol (cholest-7-en-3β-ol) which are readily interconvertible in rat intestine, *in vivo* and *in vitro*, are precursors of cholesterol rather than metabolites. Thus, if cholesterol is converted to vitamin D_3, this is a quantitatively small pathway. Quantitatively the most important route of metabolism of cholesterol is the conversion to bile acids and neutral sterols in the liver and their subsequent excretion via the bile to the intestine. It is not known in which cells of the liver, the breakdown of cholesterol occurs. The circulating cholesterol may be removed from the blood by the Kupffer cells, as on

feeding cholesterol to rats, it is found to accumulate in these reticulo—endothelial cells (Friedman, Byers and Rosenman, 1954). Interference with the function of the cells led to a reduction in this deposition. It is normally held, then, that the hepatic reticulo—endothelial system plays an important role in the removal of cholesterol from the blood and its deposition but it is not known whether breakdown of cholesterol occurs in the Kupffer cells also.

Formulae of the main bile acids occurring in bile are shown in the Fig. 1. These bile acids occur in bile as conjugates of the amino-acids glycine and taurine, to which they are bound at the carboxyl group by an amide linkage. The main bile acids in rat bile are cholic acid (80%) and cheno-deoxycholic acid (about 20%). Deoxycholic acid is formed from cholic acid in the intestine by the micro-organisms there, which remove the 7α-hydroxyl group. An active system capable of reintroducing a 7α-hydroxyl group into deoxycholic acid has been found in rat liver, the substrate being taurodeoxycholic acid. (Bergstrom and Gloor, 1954, 1955; Gloor, 1954).

When the bile acids are formed in the liver they are rapidly excreted in the bile into the intestinal lumen where they are reabsorbed and re-excreted forming an entero-hepatic circulation. Any one bile acid molecule may re-cycle fifteen to twenty times a day.

The presence of both cholesterol and bile acids in bile and the similar response of "oxy-cholesterol" (breakdown products of cholesterol) and some bile acids to a ferric chloride colour reaction suggested to Lifschütz the probably formation of bile acids from cholesterol, although the structures of these compounds were unknown at that time. "Oxycholesterol" was later found to be a mixture of the auto-oxidation products of cholesterol, 7-keto-cholesterol, and 7α- and 7β-hydroxy-cholesterol. In the period 1920 - 1930 the findings of a close structural similarity between cholesterol and bile acids seemed to lend support to Lifschütz's theory.

The first direct evidence that cholesterol was degraded in the animal organism to bile acids was not, however, produced until 20 years ago, when Bloch, Berg and Rittenberg (1943) administered deuterated cholesterol to a dog whose bile duct

drained into its bladder, thus allowing the bile to be collected and analysed. They found that the cholic acid isolated from the urine was labelled with deuterium to a highly significant degree. The finding of Bloch et al. was confirmed in 1952 by Fukushima and Gallagher, who studied the distribution in the cholesterol molecule when labelled according to Bloch. They found that about 50% of the deuterium was present in the terminal side-chain isopropyl group and as the side-chain is lost during conversion of cholesterol into bile acids, they calculated that about 85% of the cholesterol had been converted into cholic acid in Bloch's experiment.

If, then, cholesterol is converted into cholic acid, five changes in its structure must necessarily take place:-

(a) Epimerisation of the 3β-hydroxylgroup to a hydroxyl in the α-configuration.

(b) Saturation of the double bond, to give rise to the coprostane series with the hydrogen at 5 in the β-position.

(c) Addition of two hydroxyl groups in the 7α and 12α positions.

(d) Removal of the side chain to give a carboxyl group at C_{24}.

If each of these changes in structure was brought about in one enzymic step, the number of possible intermediates could be calculated. However, it is not likely that each step is a single one, and so this greatly increases the number of possible intermediates.

The sequence and detailed mechanisms of the reactions involved in the transformation of cholesterol to bile acids have not, as yet, been fully elucidated. Several techniques have been used to investigate the sequence of reactions, and these are discussed in the following paragraphs.

1. Radioactive studies

(a) Chaikoff, Siperstein and Dauben, (1952) administered intravenously, cholesterol labelled at C_{26} with Carbon^{14} to a rat and found that the isotope was expired rapidly as carbon dioxide. However, when cholesterol labelled at C_4 was administered the label was excreted in the bile acids and faeces. This evidence indicates that the steroid nucleus remains intact during its conversion from cholesterol to bile acids.

(b) Zabin and Barker (1953) concluded from the distribution of the label in the cholic acid isolated after administration of labelled acetate, that the cholic acid side-chain was derived from cholesterol by a direct removal of the three terminal carbon atoms.

2. Bile-Fistula technique

This technique has been developed and used by the Swedish workers Bergstrom (1955) Lindstedt (1957 (a)) Danielsson (1961 (b)). It consists of cannulating the bile duct of rats so that bile can be collected. Hypothetical intermediates on the pathway from cholesterol to bile acids are predicted, synthesised chemically, labelled with tritium, and injected into the rats. The bile is then collected and analysed for the presence of labelled bile acids. It should be emphasized that if the bile is completely removed from an animal in this way, profound changes will take place in the rate of bile acid synthesis which is ten times faster than in normal rats (Eriksson, 1957). This is because, when the entero-hepatic circulation is broken, no bile acids reach the liver. Bergstrom and Danielsson (1958) studied the bile acid production in bile fistula rats

during continuous infusion of taurochenodeoxycholic acid into the distal/end of the severed bile duct. As chenodeoxycholic acid does not give rise to cholic acid, cholic acid excretion could be determined. By this procedure cholic acid synthesis was reduced to the range existing in the intact rat. These results therefore indicate a homeostatic regulation of bile acid synthesis. Thus, the bile fistula rats used in these studies are abnormal.

However, by using the time taken for the conversion of cholesterol into cholic acid as a reference time, and finding how the time taken for various labelled hypothetical intermediates to form cholic acid compares with this, much useful information has been obtained by this technique.

There are two main possible pathways in the sequence of reactions from cholesterol to bile acids; either the side-chain is oxidised before any changes take place in the steroid nucleus, or the hydroxylations and other changes involving the nucleus precede the oxidation of the side-chain. In the former case, 3β-hydroxy-Δ^5-cholenic acid would be an intermediate; in the latter case coprostan-3α-7α,12α-triol (3α,7α,12α-trihydroxy-

coprostane). When these compounds were injected into bile-fistula rats, 3α,7α,12α-trihydroxycoprostane was rapidly converted into cholic acid, while 3β-hydroxy-Δ^5-cholenic acid was not (Bergstrom, Paabo and Rumpf, 1954; Bergstrom, 1955). This evidence indicates, then, that hydroxylation of the nucleus takes place before the oxidation of the side-chain is completed.

If epimerisation of the 3β-hydroxyl group to a 3α-hydroxyl and saturation of the 5-6 double bond occur before the introduction of the 7α- and 12α-hydroxyl groups, then one of the following compounds would be expected as an intermediate:

cholest-5-en-3α-ol,

coprostan-3β-ol,

coprostan-3α-ol,

cholest-4-en-3-one,

cholest-5-en-3-one.

However, none of these compounds, when injected into a bile-fistula rat was converted into cholic acid (Harold, Jayko and Chaikoff,1955; Harold, Chapman and Chaikoff, 1957; Bergstrom, 1955). Thus, hydroxylation would seem to occur before epimerisation of the 3β-hydroxyl group and possibly also the saturation of the double bond.

This assumption has been confirmed by experiments carried out by Lindstedt (1957 (b)) and Bergstrom and Lindstedt (1956), who showed that both 7α-hydroxycholesterol and also coprostan-3α, 7α-diol (3α,7α-dihydroxycoprostane) are converted into cholic and chenodeoxycholic acids in the rat, although not so efficiently as 3α,7α,12α-trihydroxycoprostane, 7α-hydroxycholesterol being the least efficiently converted.

It was also found that 3α,7α-dihydroxycoprostanic acid (Bridgwater and Lindstedt, 1960) and chenodeoxycholic acid (3α,7α-dihydroxycholanic acid) did not form cholic acid in the rat (Bergstrom and Sjövall, 1954). The former gave chenodeoxycholic acid. Thus, the introduction of the 12α-hydroxyl group apparently does not occur once the side-chain has been oxidised. This is also indicated by the fact that cholest-5-en-3β, 25-diol or cholest-5-en-3β,26-diol (25- and 26-hydroxycholesterol) do not give rise to cholic acid (Frederickson and Ono, 1956), but to chenodeoxycholic acid (Danielsson, 1961 (a)).

As it seems that many or all of the changes in the steroid nucleus take place before side-chain oxidation is completed, a possible inter

mediate is 3α,7α,12α-trihydroxycoprostanic acid, and this has been shown by Bridgwater and Lindstedt (1957) to be excreted rapidly in the bile as taurocholic acid, after intraperitoneal injection into a bile-fistula rat.

Samuelsson (1960) has shown that the inversion of the 3β-hydroxyl group probably proceeds via a 3-ketone, as cholesterol with a tritium atom in the 3α-position loses its tritium when converted into cholic acid in rat liver. He has also shown (Samuelsson, 1959) that the saturation of the double bond in the conversion of cholesterol to chenodeoxycholic acid in the pig is a stereospecific cis-addition from the β-side.

Thus, if hydroxylation at the 7-position occurs before inversion of the 3β-hydroxyl group, and also if the inversion takes place via a 3-ketone group, then cholest-4-en-3-one-7α-ol is a probable intermediate. This was suggested by Yamasaki, Noda and Shumizu (1959) from their in vitro studies (p. 24) and also by Danielsson (1961 (b)). Danielsson (1961 (b)) showed that when labelled cholest-4-en-3-one-7α-ol was injected into a bile-fistula rat, it was indeed converted into both chenodeoxycholic acid and cholic acid.

Yamasaki, Noda and Shimizu (1959) suggested that the further conversion of cholest-4-en-3-one-7α-ol to the 3α-hydroxylated compound with saturation of the double bond at the 4:5 position to give 3α,7α-dihydroxycoprostane might take place via the possible intermediate coprostan-3-one-7α-ol. Danielsson (1961 (c)) showed that this substance was indeed converted to both chenodeoxycholic and cholic acids.

The possibility that the introduction of the 12α-hydroxyl group could be one of the early steps in the breakdown of cholesterol to bile acids is not entirely remote on the evidence accumulated above. Danielsson (1961 (d)) studied the metabolism of cholest-5-en-3β,12α-diol (12α-hydroxycholesterol) in the bile-fistula rat. He found that it was converted in part to cholic acid, and pointed out that two mechanisms for the formation of cholic acid can be visualised. Firstly, 12α-hydroxycholesterol could be hydroxylated in the 7α-position before the side-chain is degraded or, secondly, the side-chain might be oxidised prior to the introduction of the 7α-hydroxy group, possibly with the intermediate formation of deoxycholic acid. If the second of these possibilities were

correct, the cholic acid formed from 12α-hydroxy-cholesterol represents only a metabolite of deoxy-cholic acid, as the latter is rapidly hydroxylated at the 7 position by rat liver. To establish the direct formation of cholic acid from 12α- hydroxycholesterol, the rabbit would be a suitable experimental animal, as in this species deoxy-cholic acid cannot be hydroxylated by the liver to cholic acid (Lindstedt and Sjövall, 1957).

It seems very likely, then, that hydroxylation at the 7-position is the initial stage in the breakdown of cholesterol to bile acids. This is suggested in the first place by the fact that the 7-position is very reactive, as is seen in auto-oxidation, and also by the fact that the "primary" bile acids contain a 7α-hydroxyl group. Also, 7α-hydroxycholesterol is converted to bile acids less readily than are 3α,7α-hydroxycoprostane and 3α,7α,12α, trihydroxycoprostane. The stereochemical course of the introduction of the 7α-hydroxyl group has been investigated by Bergstrom, Lindstedt, Samuelsson, Corey and Gregoriou (1958) with the aid of 7α and 7β-tritium labelled cholesterol. The introduction of the 7α-hydroxyl group into cholesterol during its conversion into cholic acid

occurs in a stereospecific manner, the 7α-hydroxyl group replacing the 7α-hydrogen. This indicates that the hydroxylation does not take place via 7-ketocholesterol.

3. In vitro studies

Up until about 1952 only the auto-oxidation products of cholesterol had been isolated in in vitro work.

Meier, Siperstein and Chaikoff, (1952) were able to achieve the oxidation of cholesterol labelled at C_{26} with C^{14} to $C^{14}O_2$ in rat liver slices. The same reaction has been studied in cell free preparations of mouse liver by Anfinsen and Horning (1953) and Horning, Frederickson and Anfinsen,(1957) who found that mouse liver mitochondria in the presence of NAD*, AMP and a heat-stable co-factor obtained from the cell supernatant can oxidise up to 15% of the added 26-C^{14}-cholesterol to $C^{14}O_2$. (It must be pointed out that mouse liver seems much more active in producing CO_2 from cholesterol than rat liver). Without

*The following abbreviations will be used throughout the text:-

NAD = Nicotinamide adenine dinucleotide
NADH = Reduced " " "
NADP = Nicotinamide adenine dinucleotide phosphate
NADPH = Reduced " " " "
AMP = Adenosine monophosphate
ATP = Adenosine triphosphate

this boiled supernatant factor (SF) the production of $C^{14}O_2$ from 26-C^{14}-cholesterol was much less.

This SF does not seem to be any of the well-known co-enzymes and an attempt to discover its nature was made by Horning, Frederickson and Anfinsen, (1957). The SF retained its activity for six weeks at -15°, but the activity was completely removed by passage through an acid-washed charcoal column. The activity could be recovered by elution with water. When SF was ashed no activity was observed. SF behaved on positively and negatively charged ion-exchange columns as though it contained no strong anionic groups. On paper chromatography a ninhydrin-positive fraction retaining the activity was obtained, and organic phosphate was also found to be present in this fraction. No conclusion as to the absolute nature of SF was arrived at by Horning, et al.

In Philadelphia, Gurin and co-workers (Lynn, Staple and Gurin, 1955; Whitehouse, Staple and Gurin, (1959))have investigated the same reaction using rat liver mitochondria fortified with NAD, AMP, glutathione, nicotinamide, magnesium chloride and citrate, and this SF obtained by boiling rat liver supernatant. These workers have used this

"cholesterol oxidase" system as a measure of the breakdown of cholesterol and have studied the effect of diet, sex, metal ions, etc., on this reaction.

They have reported also the conversion of 26-C^{14}-cholesterol to 25-dehydrocholesterol, to a steroid alcohol, and to an acid in these preparations, and have suggested that these might be intermediates in side-chain cleavage. They also found that this "cholesterol oxidase" system (Staple and Gurin, 1962) can oxidise coprostane-3α-7α,12α-triol 26-27C^{14} (i.e. 3α,7α,12α-trihydroxycoprostane labelled at C_{26} or C_{27} with C^{14}) to coprostane-3α,7α,12α,26-tetrol. The preparation stimulated with co-enzyme A could also cleave the side-chain with the production of propionyl CoA.

Whitehouse, Staple and Gurin, (1961) studied the distribution of SF and found that boiled particle free extracts of human placenta, bovine adrenal cortex, sow ovary, rat testes and spleen had some stimulatory activity on the oxidation of cholesterol. Boiled extracts of rat skeletal muscle, thymus, brain, kidney, blood and intestine were found to be either ineffective or inhibitory

The boiled supernatant of liver after removal of mitochondria was found to inhibit the oxidation of both cholesterol and sodium octanoate, and they suggested that this was due to calcium ions.

Frederickson (1956) investigating the products formed from cholesterol labelled at C_4 with C^{14} in the mouse liver system of Anfinsen and Horning (1955) found that of the 25% of cholesterol metabolised, 15% of the metabolic products were acids not identifiable with deoxycholic or cholic acid, 7% was 25- or 26-hydroxycholesterol and 3% was cholesterol esters.

The oxidation of 4-C^{14}-cholesterol and 26-C^{14}-cholesterol has been studied by Danielsson (1960) in mitochondrial preparations of mouse and rat liver. Again he found that addition of SF to the mouse mitochondria doubled the conversion of cholesterol to more polar products. Mouse liver mitochondria, he found, were twice as active as rat liver mitochondria. The simultaneous occurrence of autoxidation and enzymatic oxidation of the added labelled cholesterol was ascertained by analyses of the labelled products formed under non-enzymatic and "enzymatic" conditions. For non-enzymatic conditions he incubated the labelled cholesterol with SF, AMP and NAD, and for "enzymatic" conditions he used

mouse mitochondria + SF, AMP, and NAD. Among autoxidatively formed products he identified 3β,5α,6β-trihydroxycholestane, 7α-hydroxycholesterol, 7β-hydroxycholesterol and 7-ketocholesterol. Two major enzymically formed compounds (not occurring in incubations under non-enzymic conditions) both retaining the C_{27} side-chain as they were both formed from 26-C^{14} cholesterol, were isolated but not identified. One of these was found to be less polar than 7α-hydroxycholesterol, but more polar than 7-ketocholesterol. 1.8% of the added cholesterol was converted into this substance. The other product had a mobility similar to that of a neutral trihydroxy-C_{27}-sterol and was found to be converted into chenodeoxycholic acid in the bile-fistula rat. 2.5% of the added cholesterol was converted into this compound. (13% of the added cholesterol was converted into more polar products). Danielsson pointed out that there is no evidence that these "enzymatic"products are formed directly from cholesterol, as they could just as well be enzymically transformed autoxidation products, probably from 7α-hydroxycholesterol.

It can thus be seen that any *in vitro* study of the breakdown of cholesterol is complicated by the possibility of auto-oxidation taking place and it is for this reason that it is considered to be more advantageous to study the metabolism of the possible early intermediates in cholesterol breakdown, *in vitro*, rather than cholesterol itself.

Yamasaki *et al.* (1959) therefore studied the metabolism of 7α-hydroxycholesterol, which is probably the first intermediate in cholesterol breakdown, in rat liver homogenates. He found that 7α-hydroxycholesterol was converted, in the presence of NAD, to a compound which, on the grounds of its u.v. absorbing properties, was suggested to be cholest-4-en-3-one-7α-ol. On fractionating the liver homogenate he found the activity for producing this substance lay in the supernatant left after spinning down "microsomes" at 80,000 x g for 30 min. It is doubtful if this procedure would give a microsome-free supernatant. He did not, however, identify the product.

Danielsson (1961 (e)) also studied the metabolism of 7α-hydroxycholesterol, but in mouse liver homogenates only. He separated the

metabolites formed by chromatography and partly identified them. The two main metabolites in incubations fortified with NAD were cholest-4-en 3-one-7α-ol, which he identified by crystallisation to constant specific activity with the unlabelled authentic compound, and a compound more polar than 7α-hydroxycholesterol, for which the structure cholest-4-en-3-one-7α,26-diol was suggested. This substance, when administered to a bile-fistula rat, was metabolised to chenodeoxycholic acid and to some extent to cholic acid. Cholest-5-en-3β,7α,26-triol, the 27-hydroxylated derivative of 7α-hydroxycholesterol, was identified as a minor metabolite of 7α-hydroxycholesterol.

Again using mouse liver homogenates, Danielsson (1961 (a)) has identified 26-hydroxycholesterol as a metabolite of 4-C^{14}-cholesterol. Incubations of 4-C^{14}-26-hydroxycholesterol with homogenates yielded a number of different products one of these being identified as cholest-5-en-3β, 7α,26-triol. This substance was formed from 7α-hydroxycholesterol also.

A summary of the sequence of events occurring in the breakdown of cholesterol to bile acids, as far as it is known from the above evidence, is given in Figs. 3 and 4.

Hydroxylation of steroids

The steroid ring system, as has been seen above in the case of cholesterol, is not oxidised to carbon dioxide and water in animals, but undergoes enzymic reactions which degrade the side-chain possibly through the formation of hydroxy-intermediates, and which hydroxylate, oxidise, and reduce the nucleus. Steroids, in fact, can be hydroxylated at every possible position. The positions which are affected in cholesterol metabolism are at 3-, 7-, 12-, 20-, 25-, and 26-., and other positions are involved in steroid hormone hydroxylations.

As yet the actual mechanism of steroid hydroxylation is not know, but most of the enzymic systems studied appear, when characterised, to be "mixed function oxidases" acting with reduced pyridine nucleotides as electron donors (Mason, 1958). (The term "mixed function oxidase" is applied to enzymes having two interdependent catalytic activities; reduction of one atom of an oxygen molecule coupled with specific oxygenation or hydroxylation of the other atom).

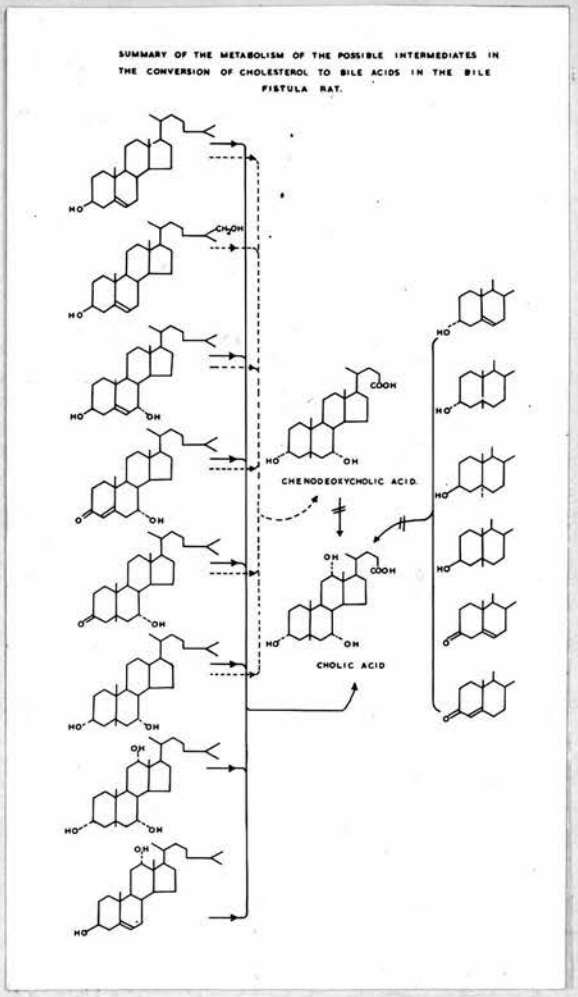

SUMMARY OF THE METABOLISM OF THE POSSIBLE INTERMEDIATES IN THE CONVERSION OF CHOLESTEROL TO BILE ACIDS IN THE BILE FISTULA RAT.
CHENODEOXYCHOLIC ACID
CHOLIC ACID

Fig. 3

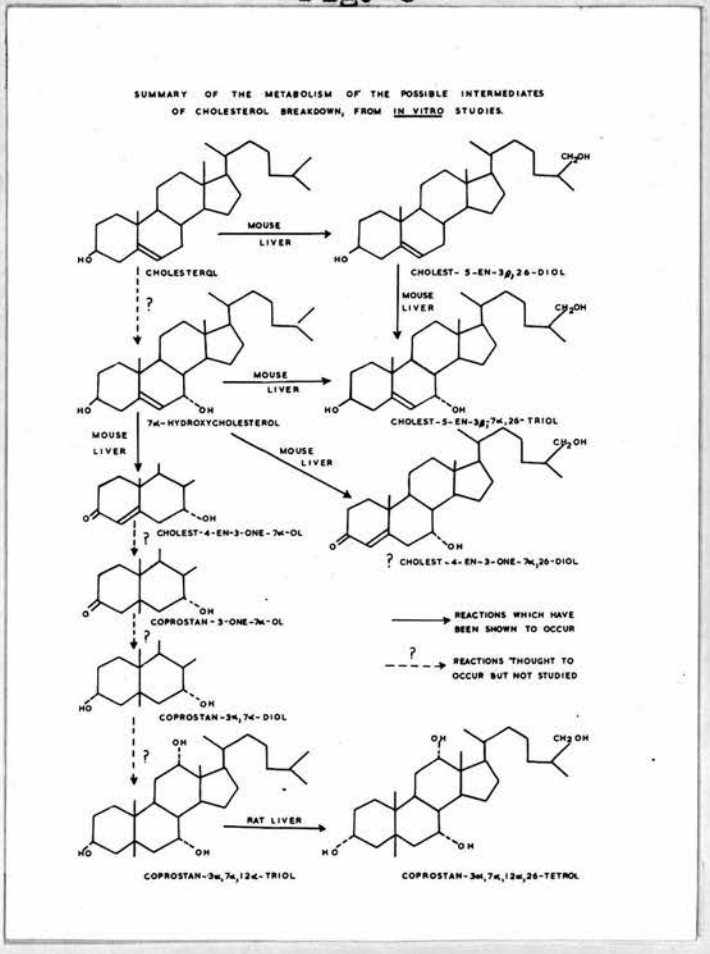

SUMMARY OF THE METABOLISM OF THE POSSIBLE INTERMEDIATES OF CHOLESTEROL BREAKDOWN, FROM IN VITRO STUDIES.
MOUSE LIVER
CHOLESTEROL
CHOLEST-5-EN-3β,26-DIOL
7α-HYDROXYCHOLESTEROL
CHOLEST-5-EN-3β,7α,26-TRIOL
CHOLEST-4-EN-3-ONE-7α-OL
? CHOLEST-4-EN-3-ONE-7α,26-DIOL
COPROSTAN-3-ONE-7α-OL
COPROSTAN-3α,7α-DIOL
RAT LIVER
COPROSTAN-3α,7α,12α-TRIOL
COPROSTAN-3α,7α,12α,26-TETROL
REACTIONS WHICH HAVE BEEN SHOWN TO OCCUR
REACTIONS THOUGHT TO OCCUR BUT NOT STUDIED

Fig. 4

Hydroxylation reactions involving the steroid hormones have been fairly extensively studied. Steroid 11-β-hydroxylation is found to be catalysed by adrenal mitochondria, but not by liver (Brownie and Grant, 1956; Grant and Brownie, 1956). Only one product is formed from deoxycorticosterone by adrenal mitochondria - the 11-β-hydroxylated compound, corticosterone - oxygen being required for the reaction. It was found that adrenal homogenates and crudely prepared mitochondria could be activated by various citric acid cycle intermediates, by ATP or NADP (Hayano, Wiener and Lindberg, 1953; Hayano and Dorfman, 1954). This situation was clarified by the discovery that NADPH is essential and specific and that the role of citric acid cycle components appears to be that of supplying NADPH (Grant and Brownie, 1956) by the sequence of reactions following:-

$$\text{Fumarate} + H_2O \longrightarrow \text{Malate} \quad \ldots \quad \ldots \quad (1)$$

$$\text{Malate} + \text{NAD} \longrightarrow \text{Oxaloacetate} + \text{NADH} \quad \ldots \quad (2)$$

$$\text{NADH} + \text{NADP} \xrightarrow[\text{transhydrogenase}]{} \text{NAD} + \text{NADPH} \quad \ldots \quad (3)$$

Tomkins, Curran and Michael (1958) found that at least two and probably three enzymes extractable from adrenal mitochondria acetone powder were required for the hydroxylation of deoxycortisone, and that the reaction was greatly stimulated by a heat-stable co-factor obtained from an acetone powder of rabbit liver. The co-factor could also be obtained from placenta, tests, and adrenal tissue. This co-factor could not be replaced by any of the following:- ascorbic acid, glutathione, nicotinamide, nicotinamide-riboside, fumarate, citrate, more NADPH, hydrogen peroxide, folic acid, or the phenylalanine-hydroxylating co-factor, (Kaufman, 1958). This "co-hydroxylase" was found to be acidic in nature.

21-hydroxylation of 17α-hydroxyprogesterone occurs in a system consisting of the combined supernatant and microsomal fractions of bovine adrenals (Ryan, 1956; Ryan and Engel, 1956). Again the reaction requires oxygen and is stimulated by NAD and ATP. Cooper, Estabrook and Rosenthal, (1963) found that addition of the steroid substrate resulted in increases in oxygen consumption and NADPH oxidation, that were equivalent to the amount of product formed. This system is similar

to the liver phenylalanine hydroxylase (Mitoma and Leeper, 1954), which has been separated into two fractions and which is activated by NADH. A co-factor from rat liver is also required and is suggested by Kaufman (1958) to be a pteridine moiety, as tetrahydrofolic acid is highly active in replacing the co-factor.

The cyclisation of squalene to lanosterol in rat liver and concomitant introduction of a hydroxyl group at position 3 is again a similar type of reaction. The oxygen of the hydroxyl group is derived from molecular oxygen (Tchen and Bloch, 1956) and the oxidative cyclisation is catalysed by microsomes together with the supernatant fraction. Apparently one of the roles of the supernatant is to provide a reductant, as it can be partially replaced by NADH or NADPH.

Thus, hydroxylation seems to be a complicated type of reaction, often requiring two enzymes, and in most cases it would seem that the supernatant fraction of the cell plays some part in the process apart from supplying NADPH.

Hydroxylations of cholesterol have not been studied to the same extent as have hydroxylations of C_{21} steroids. However, it has been suggested by Whitehouse *et al.* (1961) that the distribution of SF (see p. 21), the co-factor which activates in some way the conversion of cholesterol into

carbon dioxide, suggests a resemblance to the co-hydroxylase of Tomkins (described above) in as much as it is found in those tissues which hydroxylate and oxidise steroids.

Mason (1958) has suggested two types of mechanism for "mixed function oxidation" reactions, as all these steroid hydroxylations appear to be. In one of these, type (1), he suggests that the hydroxylating intermediate is the enzyme-oxygen complex $E\text{-}O_2$, which during hydroxylation is transformed into E-O. In a second and third stage, the electron donor reduces E-O to E and O^{2-}, as is shown below:-

Type (1) $E + O_2 \longrightarrow E\text{-}O_2$

$E\text{-}O_2 + AH \longrightarrow AOH + E\text{-}O$ (where A is the substance undergoing hydroxylation)

$E\text{-}O + DH_2 \longrightarrow E.OH + DH \longrightarrow E + D + H_2O$ (where DH_2 is the electron donor)

In the second possible type of mechanism suggested by Mason the hydroxylating intermediate is an enzyme-peroxide complex, EO_2^{2-} or $E.H_2O_2$, formed by two-equivalent reduction of enzyme-oxygen complex.

Type (2) $E.O_2 + DH_2 \longrightarrow E.O_2H_2 + D$

$E.O_2H_2 + AH \longrightarrow A.OH + H_2O$

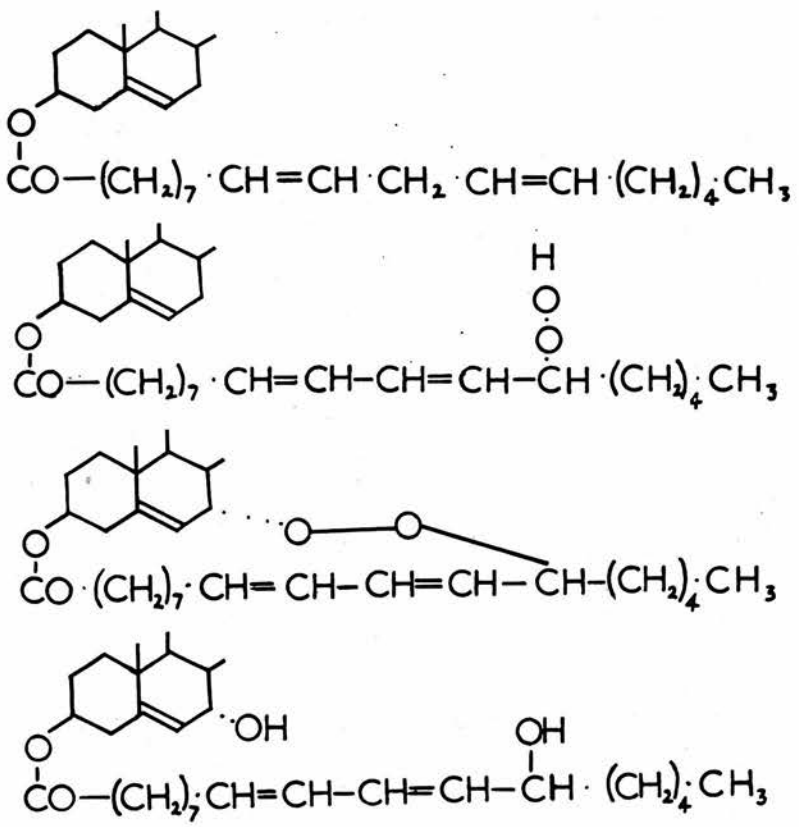


Fig. 5

Thus, one can imagine that hydroxylation of steroids might take place by one of the above mechanisms. It might be that the molecular oxygen is "activated" by formation of a peroxide, which might then be bound to the enzyme. Linoleic acid, which esterifies readily with cholesterol, can form a hydroperoxide, and it has been suggested by Mawer (1962) that the linoleic acid may play a role in 7α-hydroxylation of cholesterol. Her theory is outlined in Fig. 5.

It is also possible that hydroxylation might take place by the formation of a hydroperoxide of the steroid. Hydroperoxides of steroids have been synthesised chemically by photo-oxygenation reactions. Thus, if, for example, the 7α-hydroperoxide of cholesterol could be formed enzymically the electron donor NADPH might reduce the peroxide to form 7α-hydroxycholesterol.

Hydroxylation is not the only type of reaction common to cholesterol and the C_{21} steroid hormones. The reduction of Δ_4-3-ketones for example, is an important reaction in hormone metabolism, as it is generally thought that ring A unsaturation is important for the biological activity of the hormones, and hydrogenation of the double bond

causes inactivation. This reduction occurs primarily in the liver and has been studied extensively by Tomkins, Bakemeier and Weinberg, (1961) using cortisone as substrate. Upon reduction of the 4:5 double bond, an asymmetric centre at position 5 is produced, which means that either of two isomers, 5α or 5β, is possible, as discussed on p. 2 . The liver enzymes which specifically catalyse the production of one or other of the isomers can easily be separated by centrifugation. The 5-β reductases reside in the supernatant fraction of the cell, whereas the enzymes catalysing the formation of the 5α- reduced isomers are associated with the microsomal fraction. NADPH is required for both types of reductase. On partial purification of the 5β-reductase activity it was apparent that several reductases exist in this fraction, each of which seems to be substrate-specific.

Thus, in the supernatant fraction of rat liver, cortisone is reduced to dihydrocortisone. As the enzyme preparation usually contains 3α-reductase activity also, tetrahydrocortisone is also produced. These reactions are analogous to those which one might predict would occur if cholest-4-en-3-one-7α-ol is an intermediate in cholesterol

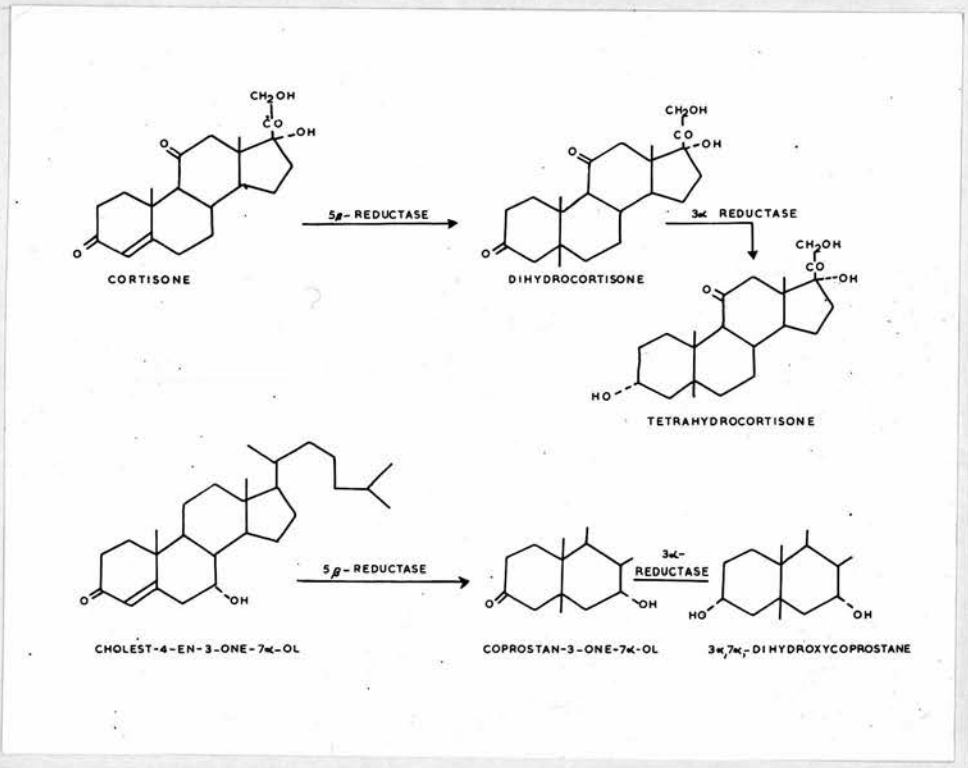

CH2OH
CO
OH
O
5β- REDUCTASE
CORTISONE
DIHYDROCORTISONE
3α REDUCTASE
HO
TETRAHYDROCORTISONE
CHOLEST-4-EN-3-ONE-7α-OL
3α-
REDUCTASE
COPROSTAN-3-ONE-7α-OL
3α,7α-DIHYDROXYCOPROSTANE

Fig. 6

metabolism, and is, indeed, reduced to 3α,7α-dihydroxycoprostane (see Fig. 6).

Outline of the work undertaken in this Thesis

From the information obtained by the techniques in the work described above, the possible sequence of events occurring the breakdown of cholesterol can be built up. This is summarised in the diagram on p. 25 .

From the evidence, it can be seen that there are many "gaps" in our knowledge in the proposed sequence of events. For example:

(a) the enzymic formation of 7α-hydroxycholesterol from cholesterol has never been demonstrated;

(b) the metabolism of 7α-hydroxycholesterol has not been studied in rat liver. Danielsson's studies on mouse liver may not give the same results as rat liver, and as the bile-fistula studies have always been carried out on rats it seems more logical to carry out in vitro work on the rat also;

(c) it has been suggested by Yamasaki that cholest-4-en-3-one-7α-ol, a metabolite of 7α-hydroxycholesterol in mouse liver, might be

reduced, possibly via coprostan-3-one-7α-ol, to give 3α,7α-dihydroxycoprostane, but there is no *in vitro* evidence to support this;

(d) the conversion of 3α,7α-dihydroxycoprostane to 3α,7α,12α-trihydroxycoprostane has not been demonstrated;

(e) the role of SF in cholesterol metabolism has not been studied. So far it has been shown that SF will increase the over-all conversion of cholesterol to either carbon dioxide or to more polar products. It has been suggested that SF may have some effect on hydroxylation reactions, but this has not been proved.

In this work an attempt has therefore been made:

(a) to study the hydroxylation of cholesterol, giving 7α-hydroxycholesterol;

(b) to study the metabolism of 7α-hydroxycholesterol in the different cell fractions of rat liver;

(c) to study the metabolism of cholest-4-en-3-one-7α-ol in the different cell fractions of rat liver and show that this substance can be converted into 3α,7α-dihydroxycoprostane;

(d) to obtain more information on the role of SF in cholesterol metabolism, and to discover whether it has an effect on any one reaction in cholesterol breakdown.

SECTION II

GENERAL PROCEDURES

GENERAL PROCEDURES

1. CELL FRACTIONATION

Animals used

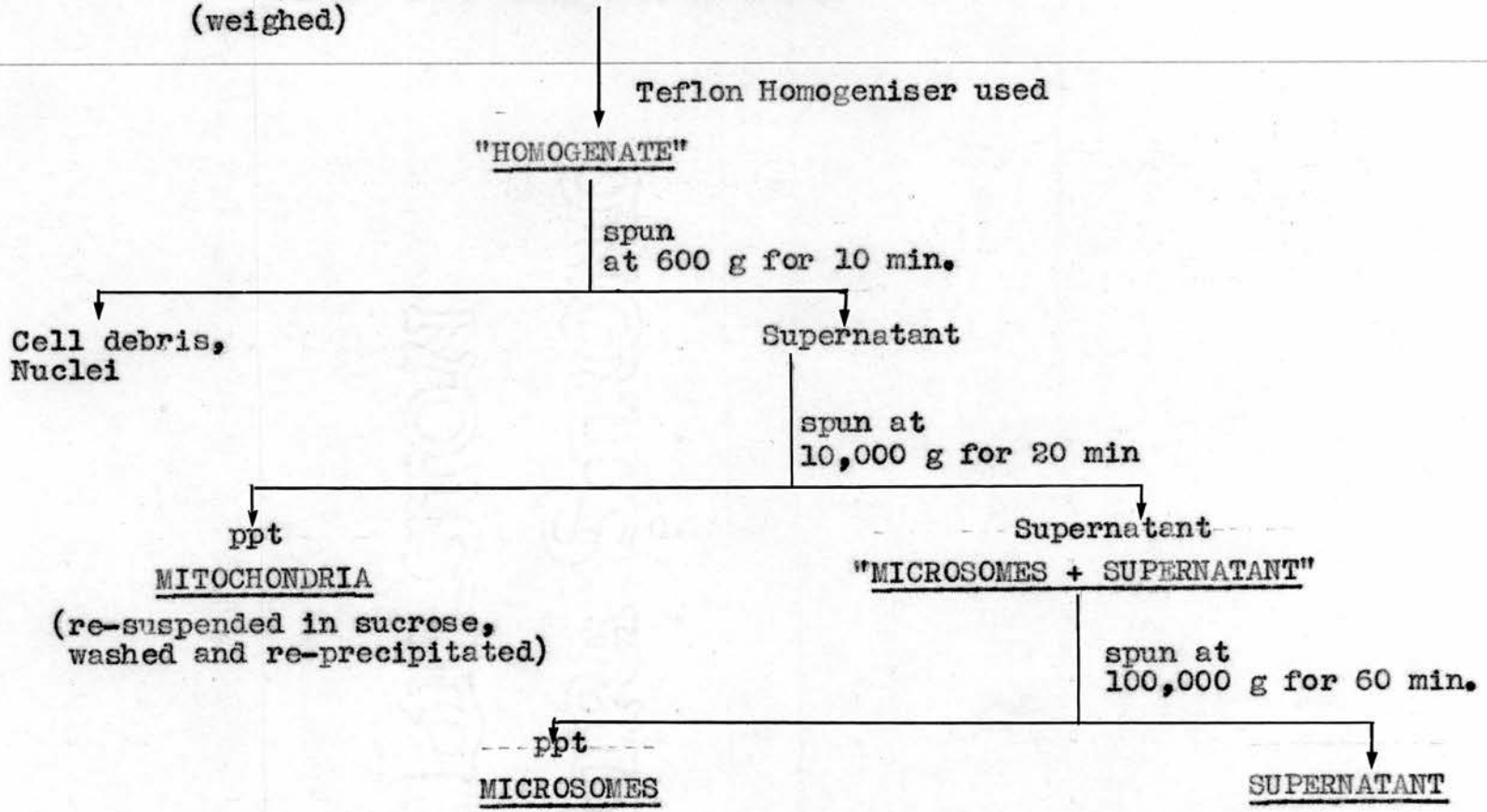
Normal rats of either sex were used in these studies. These animals had been fed on stock diet and usually weighed 150 - 200 g., yielding livers of about 9 - 12 g.

The animals were killed by a sharp blow on the head, and the liver was removed immediately and dropped into a beaker which was kept ice-cold. The liver was weighed, chopped up finely with scissors and homogenised in four times its volume of 0.25 M sucrose. A teflon homogeniser was used and all operations were carried out at 0°C.

The homogenate was then separated into cell fractions by the following procedure. Using the MSE refrigerated centrifuge, nuclei and cell debris were spun down at 600 x g for 10 min. This fraction was discarded and the supernatant, which contained mitochondria, microsomes and the soluble fraction, was spun at 10,000 x g for 15 min. This sedimented mitochondria and probably lysosomes. The mitochondria were washed with 0.25 M sucrose and re-sedimented. They

TABLE 1.

Cell Fractionation Procedure



were then made up to the required volume and homogenised very gently to ensure complete mixing.

The supernatant left after spinning down mitochondria now contained microsomes and the soluble (or supernatant) fraction. Microsomes were sedimented by spinning at 100,000 x g for 60 min. on the "spinco" refrigerated ultracentrifuge. The microsomes were made up to the required volume with 0.25 M sucrose and again homogenised gently. The soluble (or supernatant) fraction which was a clear red solution, was used in this form in incubations.

This cell fractionation is according to the method of Schneider and Hogeboom (1952).

Preparation of Boiled Supernatant Factor (SF)
(Anfinsen and Horning, 1953)

The supernatant fraction was heated at 100°C. for 10 min. The precipitated protein was removed by centrifugation, leaving a clear, yellow-brown solution, which was then stored in the deep-freeze for as long as eight weeks.

Preparation of an Acetone Powder of Mitochondria or Microsomes

The mitochondria or microsomes were prepared as above. The mitochondria were then poured very slowly, with vigorous stirring, into a large volume (500 ml.) of acetone, which was cooled by placing in a beaker of acetone and solid carbon dioxide. The microsomes, being rather gelatinous, had to be stirred with the minimum volume of sucrose before being poured into the cold acetone. The tissue formed a precipitate, which was very quickly removed by spinning, and pouring off the acetone. The tissue precipitate was extracted three or four times with ice-cold acetone, to ensure that all water had been removed. Lastly the precipitate was washed twice with ice-cold ether and then dried in a vacuum desiccator. The resulting pinkish powder could then be stored in this state in the deep-freeze and mixed with buffer when required for incubation.

2. EXPERIMENTAL CONDITIONS

Incubations of the substrates used in this study with the cell fractions of rat liver were carried out in stoppered flasks with a capacity of about 50 ml. but with relatively large, flat bases to present as great a surface area as possible to the gas phase, which in most cases was air, but on some occasions nitrogen or oxygen. The flasks were incubated in the water bath at 37°C. with shaking, for one hour, unless otherwise stated.

The incubation mixture used was the following:

200 µg. substrate (for example, 7α-hydroxycholesterol; 0.5 µ mole) in 0.1 ml. methanol

0.5 ml. 0.2 M nicotinamide (100 µ moles)

0.5 ml. 0.2 M magnesium sulphate (10 µ moles)

0.5 ml. 0.02 M phosphate buffer, pH 7.4

1 ml. 0.01 M NAD (10 µ moles)

3.0 ml. tissue (equivalent to 1 g. wet weight of liver)

As small amounts of tissue were used it was possible to set up 8 or 9 incubations on the cell fractions from any one rat liver so that each rat acted as its own control. A boiled tissue control was usually included in any set of incubations.

3. ANALYSIS OF INCUBATION MIXTURES

The investigation of the metabolism *in vitro* of cholesterol and possible intermediates in the breakdown of cholesterol to bile acids, was carried out by incubating the substrate to be studied as described, with the different cell fractions of rat liver. The substrate and metabolites produced from it during the incubation were extracted from the tissue together with a quantity of interfering lipid material. The lipid extract was therefore partially purified by a crude chromatographic column technique which separated the constituents of the extract into three groups. The final separation of substrate and metabolites was carried out by thin layer chromatography giving a method whereby the amount of substrate disappearing (i.e. metabolised) during the incubation and the amount of products formed, could be estimated.

a) Lipid Extraction

The best type of extraction was found to be one in which two layers of solvent were formed; one layer, which was organic, contained most of the lipids, and the other aqueous layer, contained all the water-soluble substances from the tissue and incubation mixture.

The reaction occurring in the incubation mixture was stopped by adding 12.5 ml. methanol. The tissue and methanol were then transferred quantitatively to a Quickfit tube, where the mixture was shaken vigorously. The protein was centrifuged down and the methanolic supernatant decanted into a further Quickfit tube. The protein was then re-extracted with 25 ml. of chloroform, and after spinning, the chloroform was added to the methanol, giving a two-phase solution. 60% saturated ammonium sulphate was added to the aqueous layer to make it more ionic and thus draw the steroids into the chloroform layer. After shaking, the mixture was centrifuged and the aqueous layer discarded. The chloroform layer was taken to dryness, in vacuo, together with a further ethyl acetate extract of this tissue protein. This extraction procedure is outlined in the diagram.

7α-hydroxycholesterol was used to check the recoveries from boiled tissue. This typical substrate was chosen, partly because much of the work in this thesis is concerned with its metabolism and partly because it can be estimated readily by the Lifschütz colour reaction, which is

described in the Appendix (p. 309). Using the above extraction procedure, together with the column method to be described, 97% of the 7α-hydroxycholesterol added to boiled tissue could be recovered. With every set of incubations a boiled tissue incubation was included. This acted as a control and indicated whether the extraction method was working satisfactorily. The recoveries of 7α-hydroxycholesterol from these controls, have been collected from all experiments performed on the metabolism of this substance and the average recovery is found to be 95% ± 3%.

This chloroform:methanol extraction procedure is a modified one, developed after it was found that recoveries of substrate using the method of Yamasaki et al. (1959) were very low indeed. The method of Yamasaki is described in the following paragraph.

To each incubation mixture was added 40 ml. of ether:alcohol (3:1) and the mixture was transferred to a stoppered tube, shaken, and boiled. The precipitated protein was spun down and the supernatant was taken to dryness in vacuo. This was a difficult procedure as the extract contained

a great deal of water which had originally been in the incubation mixture. The residue, which contained all the water-soluble substances such as magnesium sulphate, etc., was then "dissolved" in 30 ml. of ether. "Dissolved" is, of course, not the correct term because much of the residue was insoluble in ether.

When this method was checked for 100% recovery of 7α-hydroxycholesterol as a typical substrate from boiled tissue it was found to be completely unsatisfactory, only about 30% of the added 7α-hydroxycholesterol appearing in the ether solution which was columned in order to estimate this substance, at the end of the extraction. In order to find out what had happened to the 7α-hydroxycholesterol an experiment was set up in which 7α-hydroxycholesterol was added to the various constituents comprising the incubation mixture separately (Table 2). The mixtures were then treated as in the method described above, the ether extracts of the dry residues being taken to dryness and the 7α-hydroxycholesterol recovered was estimated by the Lifschütz reaction (see p.309). As can be seen from the Table, 100% recovery of 7α-hydroxycholesterol could be achieved only when

TABLE 2.

Tube Number	Contents of Tube	Water Added	µg. 7α-hydroxy-cholesterol added	µg. 7α-hydroxy-cholesterol recovered	Percentage Recovered
1	0.5 ml. Nicotinamide	3 ml.	190	190	100%
2	0.5 ml. Magnesium Sulphate	3 ml.	190	190	100%
3	3 ml. Phosphate/ Sucrose Buffer	0.5 ml.	190	60	32%
4	3 ml. Phosphate Buffer	0.5 ml.	190	190	100%
5	3 ml. 0.25 m Sucrose	0.5 ml.	190	30	15%

no sucrose was present. When sucrose was present the 7α-hydroxycholesterol was found to remain in the flask with the ether-insoluble residue. This was rather disquieting as sucrose is the best medium in which to prepare cell fractions. It may be that sucrose forms some sort of complex with 7α-hydroxycholesterol which is insoluble in ether. This difficulty was overcome, however, by employing the two phase extraction procedure described previously.

b) Separation of Lipid Extract into its Constituents

Because of the amount of interfering lipid also extracted from the tissue by this procedure, it is not possible to estimate substrates directly in the lipid extract. For example, 7α-hydroxycholesterol recovered from an incubation mixture cannot be estimated directly because of interference in the Lifschütz colour reaction by substances such as vitamin A ester.

A crude column chromatography method (Yamasaki et. al. 1959) was therefore used to separate the constituents of the lipid extract into three fractions. This was a very rapid method and was easily adapted to routine estimations on as many as ten different extracts.

The dry residues of the lipid extracts were dissolved in 10 ml. dry ether and put on to 2 g. alumina columns (Savory and Moore), prepared in ether. The columns, of dimensions 4" x ¼", were held in a row, vertically attached to a board, so that ten columns could be dealt with in turn. The columns were eluted with a further 10 ml. of ether, 25 ml. ether:alcohol (9:1) and 25 ml. alcohol. As shown in Table 3 , vitamin A ester, cholesterol esters and 7α-hydroxycholesterol esters were eluted in the ether cut, 7α-hydroxycholesterol and substances of similar polarity in the ether:alcohol cut and polar substances in the alcohol cut.

i) Using 7α-hydroxycholesterol as a typical substrate, its recovery from the 2 g. alumina column was checked. Amounts of 7α-hydroxycholesterol varying from 12 µg. to 300 µg. were taken to dryness and put on to 2 g. alumina columns in 10 ml. ether. The columns were eluted with a further 10 ml. ether, and 25 ml. ether:alcohol (9:1). The latter cuts were taken to dryness and the 7α-hydroxycholesterol recovered was estimated by the Lifschütz reaction. The results are given in Table 4 and it can be seen that 100% recovery was obtained in most cases.

TABLE 3

Elution of Substances From 2 g Alumina Columns

Alumina of Brockmann Activity II (Brockmann and Schodder, 1941)

Substance	µg. added	Ether cut (20 ml.)	Ether:Alcohol cut (25 ml.)	Alcohol cut(25 ml.)
Cholesterol Palmitate	100	+	-	-
Vitamin A Palmitate		+	-	-
7α-hydroxycholesterol laurate	100	+	-	-
Cholesterol	200	+	+	-
Cholest-4:6-dien-3-one	100	+	-	-
Cholest-4-en-3-one-	100	+	-	-
7α-hydroxycholesterol	200	-	+	-
Cholest-4-en-3-one-7α-ol	200	-	+	-
26-Hydroxycholesterol	200	-	+	-
7-ketodholesterol	100	-	+	-
7β-hydroxycholesterol	100	-	+	-
Cholest-4-en-3α,7α-diol	100	-	+	-
3α,7α-dihydroxycoprostane	100	-	+	-
3α,7α,12α-Trihydroxycorpostane	100	-	+	+ (trace)

TABLE 4.

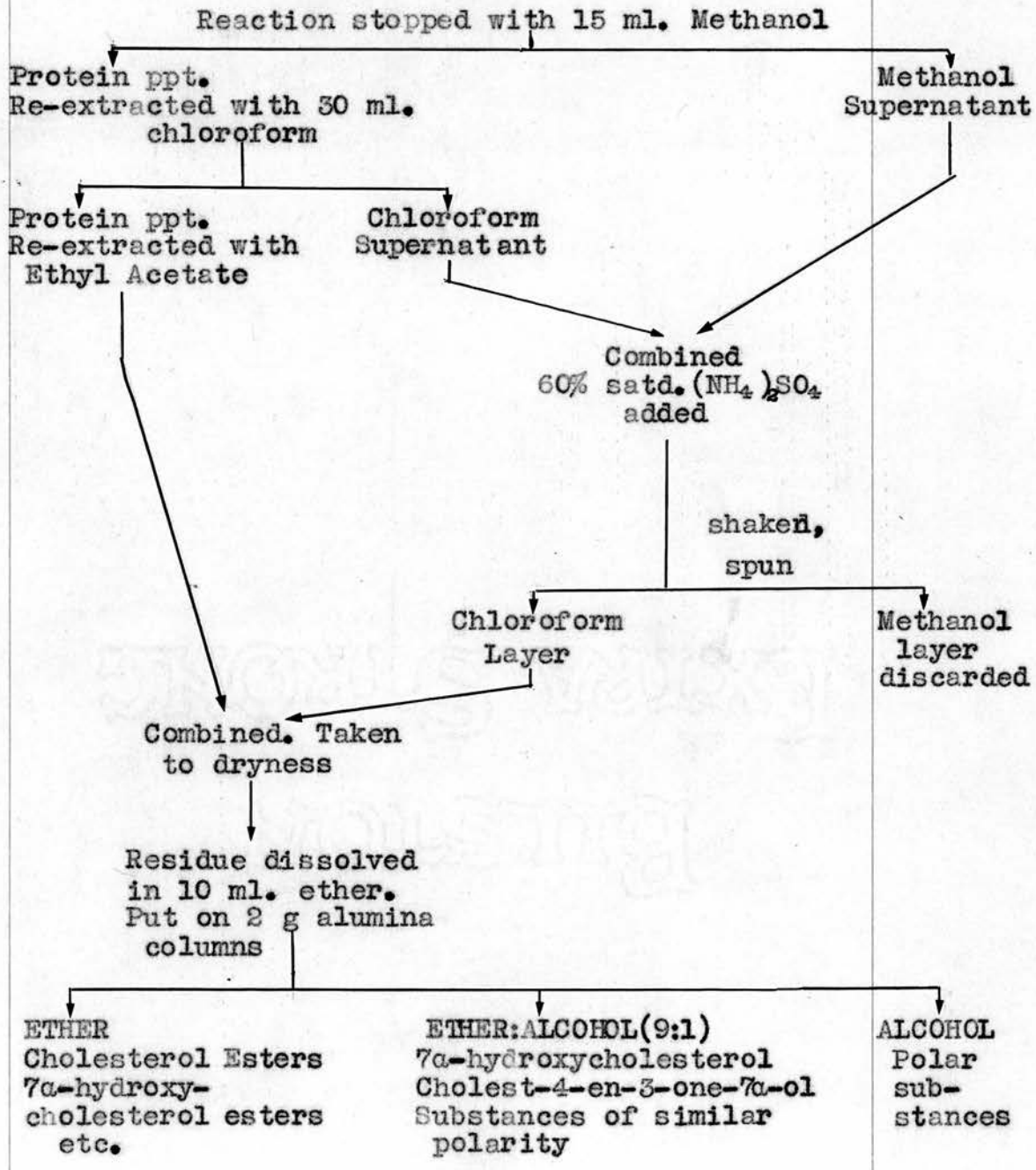
Recovery of 7α-hydroxycholesterol
From 2 g. Alumina Columns

7α-hydroxy-cholesterol added to columns µg.	7α-hydroxy-cholesterol recovered from columns µg.	Percentage Recovery
12	12	100
30	29	97
52	52	100
64	64	100
90	89	99
118	119	100
128	128	100
205	206	100
310	312	100

TABLE 5.

Extraction Procedure

5.6 ml. Incubation Mixture



Thus, substrates or metabolites, such as 7α-hydroxycholesterol, recovered either from "boiled" or "active" tissue, could be estimated free from interfering substances, by taking one half of the ether:alcohol cut to dryness and carrying out a Lifschütz colour reaction or some suitable colour test, on the residue. The other half of the cut could then be used for identification of the constituents of this cut.

It was found, however, that 7α-hydroxycholesterol was about the only compound which could be estimated directly in the ether:alcohol cut because of the specificity of the Lifschütz reaction. As will be seen, it is impossible to estimate ultra-violet absorbing substances directly.

ii) Attempt to estimate directly ultra-violet absorbing products

As one of the possible intermediates in the breakdown of cholesterol is cholest-4-en-3-one-7α-ol (see Introduction, p. 16), which is an α,β-unsaturated ketone with an absorption peak in the u.v. at 242 mμ. it was decided to study the u.v. absorption spectrum of the ether:alcohol cuts off the alumina columns to discover if any 242 mμ.-

absorbing material had been formed in incubations with 7α-hydroxycholesterol. The cuts were taken to dryness in vacuo and the residue dissolved in ethanol. The absorption spectrum of the solution was then plotted, using the Optica Recording Spectrophotometer, from 200 mμ. - 300 mμ. In order to balance the machine it was necessary to dilute the solution considerably. The solution then gave one peak at 216 mμ. and two peaks at 258 mμ. and 262 mμ. This spectrum, however, was found to be due to nicotinamide which had been added to the original incubation mixture and had been brought through the extraction procedure. Thus nicotinamide completely obliterates any other u.v. absorbing substances that may be present in the cut, and obviously, in order to identify u.v. absorbing products either the nicotinamide must be removed or a different technique must be adopted.

Attempt to remove nicotinamide

The residues from the ether:alcohol cuts were dissolved in 20 ml. chloroform and the chloroform extracts were washed three times with acid ammonium sulphate to aid the extraction of the nicotinamide into the aqueous layer, leaving the steroids in the chloroform layer. The chloroform

layers were taken to dryness and the absorption spectra of the ethanolic solutions plotted. This procedure had removed the nicotinamide, but the spectra were still very complicated and it did not seem possible to use this technique for identifying u.v. absorbing metabolites.

On paper chromatography of these residues (see p. 55) it was found that the acid-washing procedure had given rise to many products which had been absent before the procedure. For example, substances which gave a blue colour with phosphotungstic acid and which had mobilities similar to esters and di-esters of 7α-hydroxycholesterol were formed. It was thought that the acid treatment had converted nicotinamide into nicotinic acid which had esterified with 7α-hydroxycholesterol both at the 3 and 7 positions.

In order to identify and estimate metabolites other than 7α-hydroxycholesterol it is therefore necessary to separate the constituents of the ether:alcohol cuts further, as no direct estimation is possibly.

c) Further Separation of Metabolites

i) Attempt to separate metabolites by various column chromatography systems

Many types of columns were tried in an effort to separate completely the metabolites likely to be formed in incubations with 7α-hydroxycholesterol or other substrates for estimation and identification.

Attempts were made to separate the constituents of a standard mixture containing the following substances which were thought to be possible metabolites:

7α-hydroxycholesterol	200 µg.	added to each column
7α-hydroxycholesterol laurate	100 µg.	
cholest-4-en-3-one-7α-ol	100 µg.	
cholesterol	100 µg.	

Columns of alumina, silicic acid (methanol and ether washed), activated silicic acid and silica gel G were tried, using a variety of solvents. However, complete separation of these components was never achieved although partial separation was easily obtained. When the above mixture was added to boiled tissue and the lipids extracted as in a normal incubation experiment, the separation of the constituents became even

more difficult, probably due to the other lipids extracted during this procedure.

ii) Paper chromatography

Further separation of metabolites can be achieved however by concentrating the cuts off the columns and running them on paper chromatograms. Paper chromatography is not very suitable for such non-polar substances as steroids and in order for them to run at all the paper must be impregnated with paraffin. The solvent system used was chloroform:methanol paraffin (50:40:10), and after 24 hr. equilibration in the tank, the run was started, taking about 7 hr. The papers were then removed from the tank, viewed under ultra-violet light to detect u.v. absorbing substances, and then sprayed with a 10% ethanolic solution of phosphotungstic acid. On heating above a hot-plate spots developed and a list of the colours produced by various steroids with this reagent is given in Table 39, (see Appendix, p. 310).

As the paper chromatography technique is so slow and does not give reproducible results, thin layer chromatography was tried.

iii) Thin layer chromatography

This technique, reviewed by Demole (1961), involves spreading a thin layer of a mixture of silicic acid and calcium sulphate, which acts as a binder, on a glass plate. The plate is dried in an oven at 100° and left there for 20 min. to activate the silicic acid. The substances to be separated are spotted on to the plate with a capillary in the usual way. The plate is then placed in a tank or jar, in the bottom of which is the solvent. Ascending chromatography then takes place, a run taking about 40 min. or an hour. To detect substances, the same reagent is used as in paper chromatography. Phosphotungstic acid is a fairly specific reagent and in order to show up phosphotungstic acid - negative substances, the plate is sprayed again with a 10% ethanolic solution of phosphomolybdic acid which is non-specific, giving blue-coloured spots on a yellow background, but very sensitive (1 - 2 μg.).

This method has one disadvantage in that u.v. absorbing substances cannot be detected. To solve this problem, quartz plates were obtained and thin layer chromatography was carried out on these. However, the silicic acid layer absorbed or

scattered the light, so that the difference between the background and the substance was not very great.

It was then decided to add an inorganic phosphor to the silicic acid - thephosphor had to be inorganic as an organic phosphor would have moved up the plate with the solvent. The phosphor used was zinc silicate, activated with manganese. Under the light from a mercury lamp (maximum emission at 254 mμ.) the plate fluoresced a vivid green and u.v. absorbing substances showed up as dark spots (i.e. substances which absorb between 240 and 280 mμ) when viewed from the "glass" side. The spots could be marked on the back of the plate with a grease crayon (Boyd and Hutton,1963). The photograph shows some of the results obtained. (Fig.15).

It was found that substances which cannot be separated on paper chromatography can easily be separated on thin layer chromatography. The phosphor on fluorescent plates acts as an internal indicator, as u.v. absorbing substances can be located easily without using any destructive spraying method, and also a guide is given to the position of non-u.v. absorbing substances. In view of these two facts the final separation of

of metabolites from incubation mixtures can be achieved on these plates. Metabolites can be scraped off the plates, eluted, and estimated both by radioactive measurements and by chemical means. This fluorescent thin layer plate technique could be developed further by using monochromatic ultra-violet light to illuminate the plates. Thus, by varying the wavelength of the incident light, it should be possible to identify material which absorbs at any particular wavelength on the plate. This would then not only be a means of identifying the position of u.v. absorbing substance on the plate, but by finding out the exact wavelength at which it absorbed, information on its molecular structure would be obtained.

A monochromator was obtained, but not as yet, a strong enough light source, such as a xenon source and, therefore, no experiments have been carried out on this aspect.

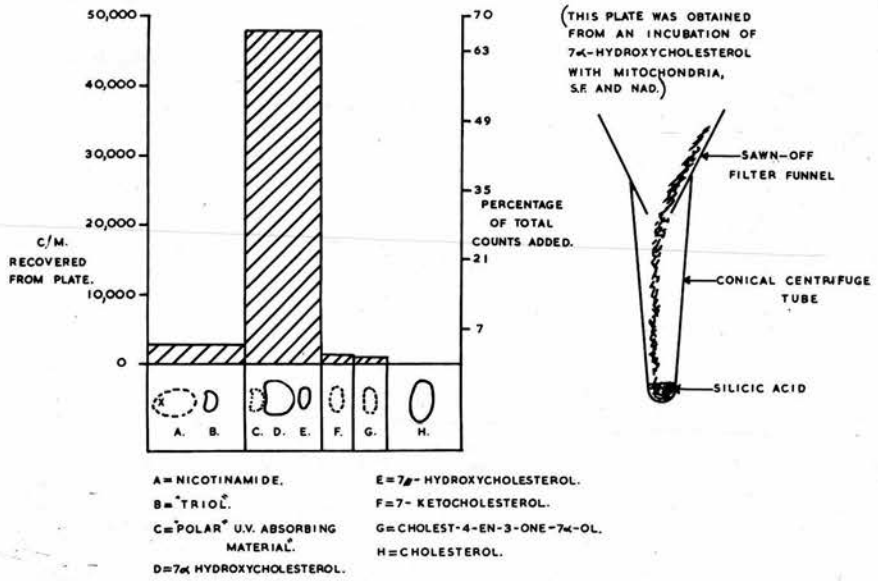
As an alternative phosphor to zinc silicate, Rhodamine G was tried. This phosphor has the disadvantage that it is soluble in alcohol and therefore for elution purposes, chloroform has to be employed. The dye is also very slightly

soluble in this solvent. Rhodamine G has the advantage, however, that non-u.v. absorbing substances can be detected under the u.v. light, as orange fluorescent spots on a yellow-pink fluorescent background. This is not very sensitive and non-u.v. absorbing material has to be present in amounts of 20 - 30 µg. before it can be detected. This phosphor was therefore only occasionally used.

Separation of metabolites on fluorescent thin-layer plates

The cuts from the 2 g alumina columns were concentrated in round-bottomed flasks in vacuo. They were then transferred to fluorescent plates, rinsing the flasks three or four times with about 0.3 ml. chloroform and transferring the rinsings to the plates as well. In this way about 95% of the contents of the flask could be put on the plates, but the "dregs" (i.e., the 5% or so left in the flasks) were always estimated either by counting, or, if the substrate was 7α-hydroxycholesterol, by doing a Lifschütz reaction in the flask. The plates were then run in the following solvent systems:

EXAMPLE SHOWING THE METHOD OF SEGMENTING A THIN LAYER PLATE AND COUNTING THE SEGMENTS



A = NICOTINAMIDE.
B = "TRIOL".
C = "POLAR" U.V. ABSORBING MATERIAL.
D = 7α HYDROXYCHOLESTEROL.
E = 7β- HYDROXYCHOLESTEROL.
F = 7- KETOCHOLESTEROL.
G = CHOLEST-4-EN-3-ONE-7α-OL.
H = CHOLESTEROL.

Fig. 9

a) for separating the constituents of the ether cut:- Benzene:dioxane (19:1)
b) for separating the constituents of the ether: alcohol cut:- benzene:ethyl acetate:acetone (10:5:3); the same solvent system, i.e. (b) was used for the alcohol cut.

When the run was completed, the plates were removed from the jars, dried, and then viewed under the u.v. light from the mercury lamp. Any u.v. absorbing spots were marked with grease crayon on the glass side of the plate. The plates were then divided into sections, usually about six, as shown in the diagram,(9)

Elution of substances from plates

Various solvents were tried, to elute a standard amount of substance from thin layer plates, including ether, ethyl acetate, chloroform, ether:alcohol (5:1) (2:1). The best recoveries were obtained with 10 ml. ether:alcohol (1:1). The silicic acid section from a plate was scraped off the plate into a conical centrifuge tube with a spatula, through a sawn-off filter funnel (Fig. 9). The silicic acid was washed into the tube with 10 ml. ether:alcohol and the silicic acid was thoroughly stirred up

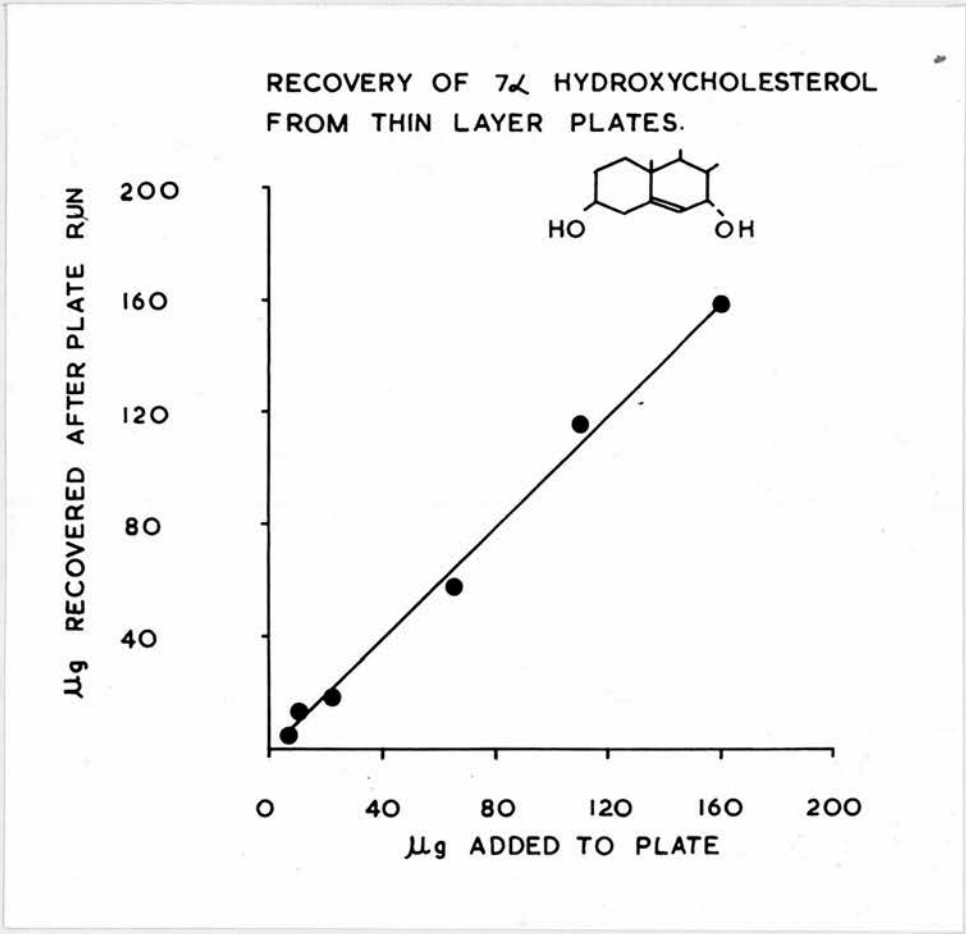

RECOVERY OF 7α HYDROXYCHOLESTEROL
FROM THIN LAYER PLATES.
HO
OH
µg RECOVERED AFTER PLATE RUN
200
160
120
80
40
0
40
80
120
160
200
µg ADDED TO PLATE

Fig. 10

TABLE 5.(a)

Recovery of 7α-hydroxycholesterol
From Thin Layer Plates

7α-hydroxy-cholesterol added µg.	7α-hydroxy-cholesterol recovered µg.	Percentage Recovery
5	4	80
10	12	120
20	17	85
63	60	95
110	114	103
160	156	97

with a thin glass rod. The silicic acid was spun down, the supernatant decanted into a Quickfit tube and taken to dryness in vacuo. The residue could then be treated in several ways:-

(a) u.v. absorbing substances

The residue was dissolved in 3 ml. methanol and its absorption spectrum plotted on the u.v. recording spectrophotometer. After this procedure the methanol could again be taken to dryness, and, if radioactive, the residue could be dissolved in 10 ml. scintillator and counted on the Packard Tri-Carb Scintillation Counter.

(b) Non u.v. absorbing substances

The residue could be used for chemical estimation. For example, 7α-hydroxycholesterol could be estimated by the Lifschütz reaction, or coprostane-3α-7α-diol by its sulphuric acid chromogen.

(c) Radioactive measurements

The residue could be counted directly as described in (a). Thus:-

1. In this way metabolites, isolated from an incubation could be estimated either chemically, or by radioactive measurements.

2. A complete radioactive analysis of the incubation mixture extract could be carried out by running each concentrated cut from the alumina columns on a plate and then segmenting the plate into sections, eluting and counting each section separately, (Fig. 9).

This procedure thus provides a powerful method of investigation, and its advantages are summarised below.

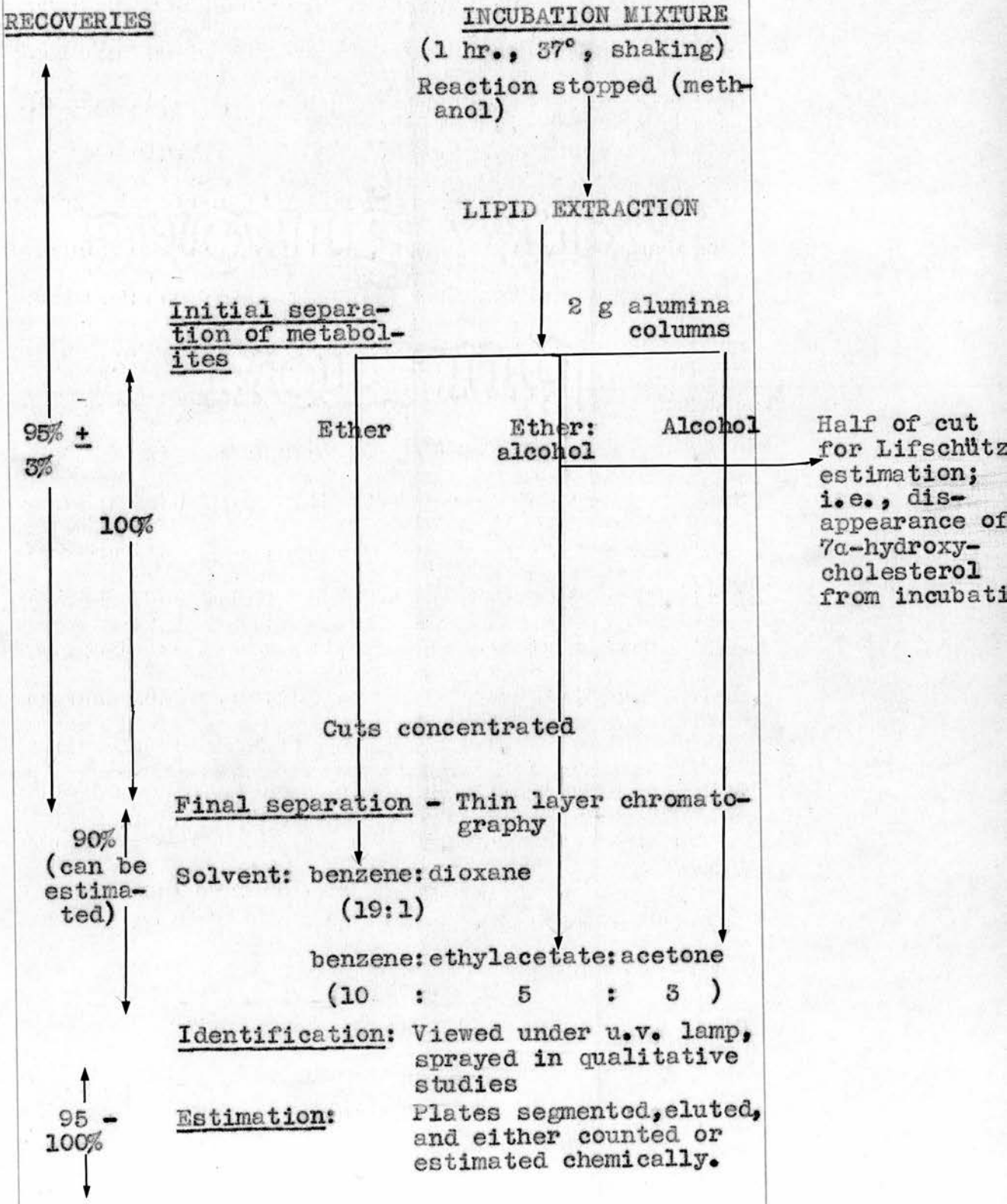
Advantages of the method

(a) Using this semi-micro method has the advantage that small amounts of substrate (100 - 200 µg.) can be incubated with tissue and the products and original substrate recovered quantitatively.

(b) As the amounts of tissue used are small, it is possible to carry out as many as eight incubations on one cell fraction of any one rat liver. Thus, each rat acts as its own control.

(c) The time taken to perform a series of incubations is only about two days, from the time of killing the rat to obtaining results.

SUMMARY OF EXPERIMENTAL PROCEDURE



SECTION III

STUDY OF THE INITIAL HYDROXYLATION OF CHOLESTEROL

STUDY OF THE INITIAL HYDROXYLATION OF CHOLESTEROL

A. Studying the initial hydroxylation at the 7α-position of cholesterol, if this is indeed the initial step, poses a very difficult problem. Because of the formation of autoxidation products, one of which is 7α-hydroxycholesterol itself (Bergstrom and Wintersteiner, 1941) it is difficult to show conclusively that the 7α-hydroxycholesterol formed has been produced enzymically. Other auto-oxidation products formed from cholesterol are 7-ketocholesterol, 7β-hydroxycholesterol and cholestane-3β,5α,6β-triol. Also any more polar products formed might arise from the auto-oxidation products, enzymically, and not directly from cholesterol. Furthermore, there is a large cholesterol pool in the liver and as no intermediates of cholesterol breakdown have ever been isolated endogenously from liver, it must be assumed that the initial hydroxylation is a rate-limiting stage. As there is so much endogenous cholesterol present in liver, it is necessary to use radioactive cholesterol in any incubations carried out to demonstrate the metabolism of cholesterol.

An experiment was therefore set up to compare the products formed from C^{14} labelled cholesterol (23 c/m/μg.) when incubated with the different cell fractions of rat liver. Active mitochondria, microsomes and supernatant were set up with C^{14}-cholesterol (430 μg. = 9890 c/m.) added as substrate, in methanol. Boiled mitochondria, microsomes and supernatant were set up as controls. No NAD was added to any incubation. The incubation flasks were incubated for 2 hr. and then the lipids were extracted and columned as described (p. 50). The ether:alcohol cuts from the alumina columns were concentrated and run on thin-layer chromatography plates. After viewing the plates under the u.v. lamp, the plates were marked off into six segments, each of which was eluted with ether:alcohol (1:1). The eluates were taken to dryness and counted.

From looking at the plates, before segmenting under the u.v. lamp it was seen that 7-ketocholesterol, an auto-oxidation product, had been formed in active and boiled mitochondria, active and boiled microsomes and boiled supernatant. Segmenting the plates and counting the sections (p.70) showed that the counts in the segments containing

7-ketocholesterol coincided with the information gained by viewing the plates in u.v. light. Thus, no difference could be seen in the counts between active and boiled mitochondria; the counts in active microsomes were slightly more (20 c/m) than in boiled microsomes, and the boiled supernatant showed more counts than the active supernatant which had not shown any 7-ketocholesterol. The possible metabolite 26-hydroxycholesterol (Danielsson, 1960) runs in the benzene:ethyl acetate:acetone system with almost the same mobility as 7-ketocholesterol and thus, if any had been produced, it would have appeared in the 7-ketocholesterol segments. It would seem, then, that none has been formed, except perhaps in active microsomes which would account for the count being higher by 20 c/m than in the boiled microsome section.

The counts in the segments which should contain 7α-hydroxycholesterol showed that none seemed to have been formed enzymically, as the counts obtained from boiled mitochondria and boiled supernatant were actually higher than these obtained from active tissue. In microsomes the counts were nearly the same in both "active" and "boiled" segments.

A further autoxidation product of cholesterol is cholestane-3β-5α-6β-triol (Danielsson, 1960). This substance would probably have a mobility causing it to run in segment (1). From the counts in these segments it would seem that this substance might have been formed in boiled microsomes. The counts in the active mitochondria segment are slightly higher than those in the boiled mitochondria, and this might be caused by the formation enzymically of a more polar substance such as the neutral trihydroxysterol found by Danielsson. The fact that 7α-hydroxycholesterol counts were lower in active mitochondria than in boiled might indicate that the 7α-hydroxycholesterol formed either by autoxidation or perhaps enzymically, has been further metabolised in the mitochondria. It is possible that it might have been converted to a more polar product (see in the fourth section of this thesis where the metabolism of 7α-hydroxycholesterol in mitochondria is discussed).

The results show that most autoxidation of cholesterol takes place in the microsomal fraction. This has been noticed many times in incubations in connection with the metabolism of other substrates. For example, in incubations with microsomes and

7α-hydroxycholesterol much more 7-ketocholesterol and 7β-hydroxycholesterol are formed than in an equivalent incubation with the other cell fractions.

The ether cuts off the alumina columns were run on thin layer plates in the benzene:dioxane system. The plates were then divided into two segments, one containing cholesterol and the other containing cholesterol esters. The segments were eluted and counted. From the results it can be seen that no esterification seems to have taken place.

It can be concluded then that hardly any, if any, enzymic metabolism of the added cholesterol has taken place. Autoxidation products have been formed, particularly in the microsomal fraction. In this experiment, the reason for failure to form any metabolic products may be due to several factors.

(a) The cholesterol added was probably not sufficiently labelled to show up any metabolic process.

(b) No additions, such as NAD, ATP, or citric acid cycle intermediates were present.

(c) The incubation time may not have been long enough for the labelled cholesterol to equilibrate with the large cholesterol pool.

(d) Addition of SF (boiled supernatant) might be required.

INCUBATION OF C^{14}-CHOLESTEROL WITH DIFFERENT CELL FRACTIONS

430 µg. C^{14}-Cholesterol added to each = 9890 c/m

Cell fraction	Mitochondria		Microsomes		Supernatant	
	Boiled c/m	Active c/m	Boiled c/m	Active c/m	Boiled c/m	Active c/m
Ether:Alcohol cut						
1. Origin	4	18	156	20	10	14
2. 7α-hydroxy-cholesterol	66	36	126	133	54	28
3. 7-ketocholesterol	40	40	173	196	130	18
4.	40	20	1154	146	141	238
5. Cholesterol	646	670	1540	2404	6164	4168
6. Less polar substances	0	6	71	135	48	29
Ether cut						
1. Cholesterol	9650	8180	6090	6870	3390	4500
2. Cholesterol esters	64	56	76	78	37	69

B. As has been discussed in the Introduction (p. 31), it is possible that hydroxylation could take place with the intermediate formation of a hydroperoxide of the steroid. Thus, the initial hydroxylation of cholesterol at the 7α-position might occur through the 7α-hydroperoxide of cholesterol (cholest-5-en-3β-ol-7α-hydroperoxide) as an intermediate. This substance might then be readily reduced by NADPH to yield 7α-hydroxycholesterol.

The 7α-hydroperoxide of cholesterol has been prepared by a photo-oxygenation method by Mr. Naqui (see Appendix, p.305). This substance on thin layer chromatography in the benzene;ethyl acetate:acetone system runs with an R_F value of 0.58. On spraying with phosphotungstic acid it gives a purple colour, and when sprayed with a solution of potassium iodide in acetic acid it appears as a yellow spot, due to liberated iodine.

Some very preliminary experiments were carried out using the 7α-hydroperoxide of cholesterol as substrate.

(i) Four incubations were set up, using 150 μg. of the hydroperoxide in 0.1 ml. methanol. Two incubations contained a solution of an acetone

powder of mitochondria in buffer, one with NADPH-generating system added, and the other without it. The other two incubations contained buffer, and one contained NADPH-generating system. The NADPH-generating system was that used on p.188 (viz., 0.1 ml. NADP (0.0048 M), 0.1 ml. glucose-6-phosphate (0.035 M), 0.05 ml. glucose-6-phosphate dehydrogenase, where 0.1 ml. is equivalent to 1 unit, where 1 unit reduces 1μmole NADP to NADPH). After incubating for 1 hr. the lipids were extracted as usual and the lipid extract was concentrated and run on thin layer plates. A column procedure was not necessary. The plates were viewed under the u.v. light and then sprayed with phosphotungstic acid.

The results are shown in the Table.

Incubation 150 μg. substrate added to each	7-keto-cholesterol formed	7α-hydroxy-cholesterol formed
(a) Buffer; NADPH	++	++
(b) Buffer; acetone powder	++	+++
(c) Buffer; acetone powder; NADPH	++	+++
(d) Buffer	++	++

As can be seen, 7-ketocholesterol, an autoxidation product, was formed in all cases approximately to the same extent. 7α-hydroxycholesterol was also formed in all cases, even when the hydroperoxide was incubated with buffer alone. By eye, it was judged that more 7α-hydroxycholesterol had been formed in the incubations involving the acetone powder. In incubations (a) and (d) which did not contain acetone powder, about one-third of the hydroperoxide added was seen to be unchanged, whereas in incubations (b) and (c) no hydroperoxide was recovered at all.

This experiment was repeated quantitatively. The incubations were set up as before, extracted, and run on plates. The 7-ketocholesterol formed in each case was marked after inspection under the u.v. lamp and eluted with ether:alcohol (1:1). The eluate was taken to dryness and the absorption spectrum of the ethanolic solution was plotted. A peak at 238 mμ. was obtained in each case and from each reading at 238 mμ. the amount of 7-ketocholesterol formed was calculated. The portion of thin layer plate containing 7α-hydroxycholesterol was also eluted in each case, and the 7α-hydroxycholesterol formed was estimated by the

Lifschütz reaction. The results are given in the Table.

Incubation 150 µg. substrate added	7-keto-cholesterol formed		7α-hydroxy-cholesterol formed	
	Optical density at 238mµ.	µg.	Optical density at 610mµ.	µg.
(a) Buffer;NADPH	0.67	48	0.08	24
(b) Buffer; acetone powder	0.84	60	0.156	47
(c) Buffer;acetone powder;NADPH	0.754	54	0.143	43
(d)Buffer	0.69	49	0.07	21

As can be seen, the amount of 7-ketocholesterol formed is approximately the same in each case, although the amount formed in incubations involving the acetone powder is slightly higher. The amount of 7α-hydroxycholesterol formed, on the other hand, in (b) and (c) is about twice that formed in the incubations without acetone powder. Adding NADPH-generating system seems to make no difference to the products formed.

These experiments show that the 7α-hydroperoxide of cholesterol is not a very stable compound under the conditions employed in an incubation as even incubating it at 37° with buffer leads to the formation of both auto-oxidation products, 7-ketocholesterol and 7α-hydroxycholesterol. Also, it is shown that acetone powder of mitochondria has an effect on the production of 7α-hydroxycholesterol. As about the same amount of 7-ketocholesterol is formed with acetone powder as without, it appears that the increased production of 7α-hydroxycholesterol may be enzymic. A control incubation containing boiled acetone powder would have to be included to settle the question of whether the 7αhydroxycholesterol was being formed enzymically.

(ii) As the 7α-hydroperoxide of cholesterol is so unstable it seemed likely that it would also form autoxidation products when subjected to the procedure required for extraction and separation of metabolites following an incubation with native mitochondria or microsomes, etc.

An experiment was set up to find the effect of incubating the hydroperoxide with 3 ml. boiled and therefore, inactive, homogenate of rat liver. 200 µg. of the hydroperoxide were incubated with boiled tissue, buffer, nicotinamide and magnesium sulphate as in a normal incubation. The lipid was extracted and the extract run on a 2 g alumina column. At the same time a further 200 µg. of hydroperoxide were put on to a similar alumina column. The ether:alcohol cuts from each column were concentrated and run on thin layer plates. On examination of the plates under the u.v. light and after spraying, it was found that in both cases 7-ketocholesterol, 7β-hydroxycholesterol, and 7α-hydroxycholesterol were formed. ( ). Thus, even passing the hydroperoxide down a 2 g. alumina column causes it to be converted to these three products. Very little hydroperoxide remained unchanged during this procedure.

As a column method is convenient to remove much interfering material from a lipid extract obtained from an incubation with native tissue, it was decided to find out whether a silicic acid column method would still give rise to the autoxidation of the hydroperoxide.

(iii) A 2 g. silicic acid column was therefore set up and a mixture of cholesterol (200 µg.), 7α-hydroxycholesterol (200 µg), and 7α-hydroperoxide of cholesterol (200 µg.) was put on to the column in a mixture of 15% ether: 85% petrol ether (60° - 80°). The column was then eluted as shown, the cuts concentrated and run on thin layer plates to identify the constituents. The results are given in the Table.

Cut No.	Solvent	Vol. in ml.	Constituents
1	15% ether: 85% petrol ether	10	-
2	20% ether: 80% petrol ether	10	Cholesterol
3	25% ether: 75% petrol ether	10	Cholesterol
4	50% ether: 50% petrol ether	10	7α-hydroxycholesterol, 7-ketocholesterol,
5	100% ether	10	7α-hydroperoxide
6	50% ether: 50% methanol	10	7α-hydroxycholesterol, 7-ketocholesterol.

As can be seen, 7-ketocholesterol is formed from 7α-hydroperoxide (7α-hydroxycholesterol and cholesterol are quite stable on silicic acid) even on silicic acid. However, only traces of 7-ketocholesterol were formed, and it was therefore decided to use this column method to remove interfering material from lipid extracts of an acetone powder of mitochondria.

(iv) Two incubations were set up each containing 100 μg. 7α-hydroperoxide and a buffer solution of an acetone powder of mitochondria. The buffer solution added to one incubation had previously been boiled and it was therefore enzymically inactive. The other incubation contained active mitochondrial powder. After incubation and extraction, the dry residues were put on to 2 g. silicic acid columns in 15% ether: 85% petrol ether. The columns were eluted with 10 ml. of this solvent, followed by 10 ml. of 25% ether: 75% petrol ether. Further cuts of 30 ml. ether:methanol (9:1) were taken. These latter cuts were taken to dryness and run on thin layer plates.

It was found that 7-ketocholesterol had been formed in both incubations, but, by eye, the amount was judged to be about the same in both cases. The portions of the plates containing 7α-hydroxycholesterol were eluted and the eluates taken to dryness. A Lifschütz reaction was carried out on each eluate and it was found that the amount of 7α-hydroxycholesterol formed in the incubation with active acetone powder was double that formed in the boiled control incubation, i.e. 20 μg. 7α-hydroxycholesterol was formed in the "active" incubation and 9 μg. in the "boiled" one. Thus, it would seem that some enzymic activity is present in an acetone powder of mitochondria which will convert the 7α-hydroperoxide of cholesterol into 7α-hydroxycholesterol. 7-ketocholesterol appears to be formed non-enzymically.

C. Experiments with Macrophages. As has been pointed out, the problem of autoxidation makes the study of the initial stages in the breakdown of cholesterol very difficult. As the autoxidation seems to be marked by the presence of large amounts of tissue, the microsomal fraction being particularly bad, it was wondered whether working

with reticulo-endothelial cells would be a "cleaner" procedure and less prone to autoxidation. It may, in fact, be the case that cholesterol degradation occurs in the liver in the Kupffer cells, which are reticulo-endothelial cells. The Kupffer cells are probably involved in the uptake of cholesterol from the blood. It would therefore be of interest to study cholesterol breakdown in preparations of macrophages, which are also phagocytic cells.

Very little is known about the metabolism of cholesterol in macrophages, but Schonheimer and Yuasa (1929) and Tompkins (1946) have found that cholesterol introduced into the subcutaneous tissues is partly converted to cholesterol esters, presumably by the macrophages present there. More recently Day and French (1959) have shown that when cholesterol suspensions are ingested by reticulo-endothelial cells in the lymph nodes of the rat, the content of cholesterol esters increases in the nodes. Day (1960) also showed that both synthetic and hydrolytic cholesterol esterase activity is present in isolated rabbit macrophages. Homogenates of these cells esterified the cholesterol at pH 6.0 and hydrolysed the esters at pH 7.3.

Day (1961) compared the oxidation by rabbit macrophages of cholesterol-26-C^{14} to $C^{14}O_2$ with the oxidation of C^{14}-labelled fatty acids (sodium palmitate-1-C^{14}) and C^{14}-labelled triglycerides. He found appreciable oxidation of fatty acids and triglycerides brought about by macrophages, but under the same conditions no oxidation of the cholesterol side-chain could be demonstrated. He found that the cholesterol was phagocytosed and thus the failure to oxidise it cannot be explained by a failure in its uptake.

It is possible, however, that the macrophages can bring about the earlier stages in degradation of cholesterol, such as the hydroxylation at position 7, whereas the splitting off of the side-chain to form bile acids may be a very complicated process for which macrophages are not equipped.

(a) Procedure for obtaining rat macrophages

Macrophages were grown by the following procedure by Dr. A.E. Stuart, Pathology Department, University of Edinburgh. "199" tissue culture medium containing heparin was injected intraperitoneally into 5 rats to wash out the macrophages accumulated in the peritoneal cavity.

The peritoneal fluid was then taken off after 5min. &usually amounted to about 26 ml. from 5 rats. A count of the number of cells in the fluid was then made and normally this was about 5 x 10^6 cells per ml., which is regarded as a reasonably high value. The required number of cells (16 x 10^6 cells/bottle) were then grown in "baby bottles" containing 8% rat serum in "199" tissue culture medium. "Baby bottles" are used because they have eight flat surfaces, and if placed sideways on one of these surfaces, the cells grow as a layer on the surface of the bottle. If the cells were seen to be growing satisfactorily in a good monolayer after 48 hr., C^{14}-labelled cholesterol in rat serum was added as a substrate. The bottles were then incubated for varying times at 37°.

(b) Extraction and separation procedure

After incubating the macrophages with C^{14}-cholesterol for the required time, methanol was added to the bottle to kill the cells and stop any enzymic reactions. Carrier cholesterol and 7α-hydroxycholesterol were added. The methanolic solution was centrifuged to remove the denatured

protein and the supernatant was decanted off. The protein was re-extracted with chloroform and the chloroform extract was added to the methanolic supernatant. After shaking and centrifuging the aqueous methanolic layer was discarded. The chloroform layer was taken to dryness and as the extract was "clean" and free from interfering substances, no column method was required. The extracts were run directly on thin layer plates, one half being run on one side of the plate and the other half on the other side. After the run, the plates were viewed under the u.v. light and any u.v. absorbing substances were marked. One half of the plate was then sprayed with phosphotungstic acid, the other half of the plate being shielded with a sheet of glass. The unsprayed half of the plate was then segmented as shown, the segments eluted with ether:alcohol (1:1) and the eluates taken to dryness and counted.

(c) Experiments with C^{14}-cholesterol and macrophages

Rat serum was obtained from rats which had been injected with C^{14}-labelled mevalonate - a precursor of cholesterol. C^{14}-labelled cholesterol and cholesterol esters thus appeared in the

blood as lipoprotein complexes. Cholesterol and cholesterol esters are therefore being presented to the macrophages solubilised in an ideal way.

Experiments were set up in which C^{14}-cholesterol (and esters) were incubated with macrophages for varying times before the reactions were stopped. The results were obtained as described above, and are set out in the Tables, (7-10).

As can be seen, the results varied considerably from experiment to experiment and from time to time. In other words there seemed to be no increased formation of more polar products as time increased. In some cases no metabolism of cholesterol appeared to have occurred at all, whereas in other cases more polar products were definitely produced.

(i) Experiment 1

Two 24 hr. duplicate incubations were set up and the results are shown. In the second incubation radioactivity in the 7α-hydroxycholesterol segment was seen, being three times that occurring in the cholesterol segment. No.7-ketocholesterol was seen on the plate before segmenting and the radioactivity present there might be due to the

TABLE 7

	Segment (c/m)		Origin (1)	7α-hydroxy-cholesterol (3)	7-keto-cholesterol (4)	Cholesterol (5)	(6)	Cholesterol esters (7)	Ratio (choles./esters)	Ratio (choles./7α)
	Time in hr.									
1)	24	CA	1147	18	42(-ve)	197		362	1:2	10:1
	24	CA	105	1620	26(-ve)	531		60	9:1	1:3
2)	Zero	CA	54	17	-	118	10	737	1:6	6:1
	6	CA	42	24	4	38	90	70	1:2	1:1
	12	CA	32	10	10	38	14	110	1:3	4:1
	21	CA	12	10	68	42	30	785	1:19	4:1
	27	CA	10	-	62	50	-	262	1:5	
3)	Zero	CA	0	10	5	40		75	1:2	4:1
	Zero	NC	18	10	10	20		85	1:4	2:1
	12	CA	56	153	30	26		50	1:2	1:6
	12	NC	35	38(+ve)	-	60		180	1:3	2:1
	24	CA	42	20	30	12	20	60	1:5	1:2
	24	NC	20	10(-ve)	15	50	126	145	1:3	5:1
	36	CA	45	20	7	50	30	160	1:3	2:1
	36	NC	27	15(+ve)	2	26	0	100	1:4	2:1

CA = carrier added (+ve) substance identified by chromatography

NC = no carrier added (-ve) none of this substance identified

formation of a substance of similar polarity, such as 26-hydroxycholesterol. In the first incubation a high count (six times that seen in the cholesterol segment) was found in the origin segment. This indicates that either polar products had been formed or that 7α-hydroxycholesterol had run in this segment. In this experiment, one half of the plate was not sprayed, as has been described, and therefore the c/m are higher than those obtained in other experiments where only one half of the extract was counted, the other half being sprayed for identification of the constituents. In this 24 hr. experiment, then, considerable metabolism of cholesterol or cholesterol esters seems to have occured.

(ii) Experiment 2

In this experiment a zero hour incubation was included as a control. Not much metabolism of cholesterol or its esters seems to have occurred in any incubation. In the 6 hr. incubation, the radioactivity in the 7α-hydroxycholesterol segment indicates that some had been formed. Esterification seems to have occurred in the 21 hr. incubation, and hydrolysis of esters in the other

incubations. The formation of 7-ketocholesterol increases with time. Thus the incubations in this experiment are rather inactive.

(iii) Experiment 3

In this experiment duplicate incubations were carried out. To one incubation extract carrier cholesterol and 7α-hydroxycholesterol were added. No carrier was added to the duplicate extract. Thus, on spraying one half of the plate on which the "no carrier" incubation had been run it was possible to see whether any 7α-hydroxycholesterol had been formed. If any was seen to be formed a + sign is placed on the Table after the c/m in that segment.

In the 12 hr. incubation, 7α-hydroxycholesterol was seen on the "no carrier" plate and the radioactivity in the corresponding segment indicates that it was produced from cholesterol or its esters. The duplicate 12 hr. "carrier added" incubation also shows a high count in this segment. 7α-hydroxycholesterol was seen also in the 36 hr. " no carrier" incubation. In all incubations except the zero hour control incubations, the radioactivity in the origin segments

is fairly high, indicating the formation of more polar products than 7α-hydroxycholesterol. 7-ketocholesterol was not seen in any of the incubations and esterification seems to have occurred in most incubations.

In this experiment, then, the 12 hr. incubation appears to have been quite active at metabolising cholesterol. The time of incubation does not seem to be important in the formation of products from cholesterol.

(iv) Experiment 4

In the 6 hr. and 15 hr. "no carrier" incubations in this experiment, 7α-hydroxycholesterol was seen to be present and the radioactivity, especially in the 15 hr. incubations, indicates that it was produced from cholesterol or its esters. However, 7-ketocholesterol was also seen to be formed and was labelled, and therefore the 7α-hydroxycholesterol may have been formed by autoxidation. Polar products would seem to be formed in the 15 hr. and 23 hr. incubations. On the sprayed half of the plates from 15 hr. "no carrier", 15 hr. "carrier" and 23 hr. "carrier" incubations, esters of 7α-hydroxycholesterol

TABLE 8

Segment (c/m)		Origin (1)	(2)	7α-hydroxy cholesterol (3)	7-keto-cholesterol (4)	cholesterol (5)	cholesterol esters (6)	Ratio choles. esters	Ratio choles. 7α-hydroxy cholesterol
Time in hr.									
Zero	CA	10	24	0	70	1160	2220	1:2	
6	CA	9	0	10	0	1145	2725	1:2.5	100:1
6	NC	5	0	15(+ve)	0(-ve)	1165	3130	1:3	70:1
15	CA	316	200	140	170	2240	2375	1:1	15:1
15	NC	200	87	50(+ve)	224(+ve)	1300	2650	1:2	25:1
23	CA	195	75	125	434(+ve)	1640	3030	1:2	13:1
Serum (NC)		70		0(+ve)	0	126	5220	1:4	

Pooled Extracts (except zero hr.) Benzene:dioxane system

Segment	7α-hydroxy cholesterol	cholesterol	7α-hydroxy-cholesterol esters	7α-hydroxy-cholesterol esters	cholest. esters
(c/m)	440	2390	235	210	4150
Identification on chromatography	+ve	+ve	+ve	+ve	+ve

CA = carrier added (+ve) shows substance identified by chromatography

NC = no carrier added (-ve) shows none of substance identified.

were seen to be present. Therefore the remainder of the extracts not put on to plates, except for the control extract, were pooled and run in the benzene:dioxane system. Again one half of the plate was sprayed and the other half segmented and counted. The segments containing 7α-hydroxycholesterol and its esters contained about one-fifth and one-tenth, respectively, of the radioactivity occurring in the cholesterol segment.

As a check 0.5 ml. of the serum used as a substrate for these incubations was extracted, run on a plate, and one half sprayed and the other half segmented and counted. A trace of 7α-hydroxycholesterol was seen to be present, but it gave a negligible count.

Thus, it would seem that metabolism of cholesterol or its esters has occurred, particularly in the 15 hr. incubations to give 7α-hydroxycholesterol, 7α-hydroxycholesterol esters, and more polar products.

(v) Experiments 5 and 6

In these experiments again, in some incubations no metabolism seems to have occurred at all.

TABLE 9

Segment (c/m)	Origin	7α-hydroxy cholesterol	7-keto-cholesterol	Cholesterol	Cholesterol esters	Ratio ($\frac{\text{choles.}}{\text{esters}}$)	Ratio ($\frac{\text{choles.}}{\text{7α-hydroxy cholesterol}}$)
5) Time in hr.							
Zero	40	20	10	140	320	1:2	7:1
3	0	20	20	350	125	3:1	17:1
6	60	40	80	480	2330	1:5	11:1
36	120	470	0	790	1810	1:2.5	2:1
46	50	10	300	490	2830	1:5	50:1
6)							
Zero	34	0	85	150	1435	1:10	∞
2	100	10	90	190	1640	1:8	19:1
5	10	20	20	100	810	1:8	5:1
21	30	30	43	40	190	1:5	1:1
24	30	0	26	170	1460	1:8	∞

Carrier cholesterol and 7α-hydroxycholesterol added to each incubation

However, in the 36 hr. incubation, 7α-hydroxycholesterol and more polar products are formed, the radioactivity in the 7α-hydroxycholesterol segment being about one-half of that in the cholesterol segment. After 46 hr. much 7-ketocholesterol appears to be produced. In the 24 hr. incubation more polar products are formed.

(vi) Experiment 7

Although Day (1961) has found that no oxidation of the side-chain of cholesterol can be carried out by rabbit macrophages, it was thought to be of interest to discover whether the more polar products obtained in some incubations in previous experiments could be bile acids.

Six incubations were prepared, and one of these was stopped at zero time. The other five bottles were incubated for 24 hr., the contents were pooled and extracted as usual with methanol. One-tenth of the methanol extract was treated as usual and was run on a thin layer plate, half of which was sprayed and the other half segmented and counted. The other nine-tenths was extracted as follows to discover whether any bile acids had been formed. The protein was spun down and the

methanol extract taken to dryness in vacuo. The residue was dissolved in sodium bicarbonate and poured into a nickel crucible. 5 N sodium hydroxide was added and the mixture was hydrolysed under pressure for 3 hr. The mixture was then acidified with concentrated hydrochloric acid and extracted four times with chloroform:ethanol (9:1). The chloroform layer was taken to dryness in vacuo and acetic acid and celite were added to the residue. A column of celite prepared in 70% acetic acid and 100% petrol ether was made, and the celite, on which the extracted material had been adsorbed, was placed on top of the column. The column was eluted as shown in Table 10 one half of each cut being taken for counting and the other half for thin layer chromatography.

In Table 10 it can be seen from the plates that more polar products have been formed, which are radioactive. In the origin segment, 26 c/m are found, and it must be remembered that this only represents about one-twentieth of the total 24 hr. incubation. Radioactivity was eluted in cut 1 of the bile acid column. This would be due to cholesterol and cholesterol esters. Some radioactivity was also eluted in the dihydroxy-

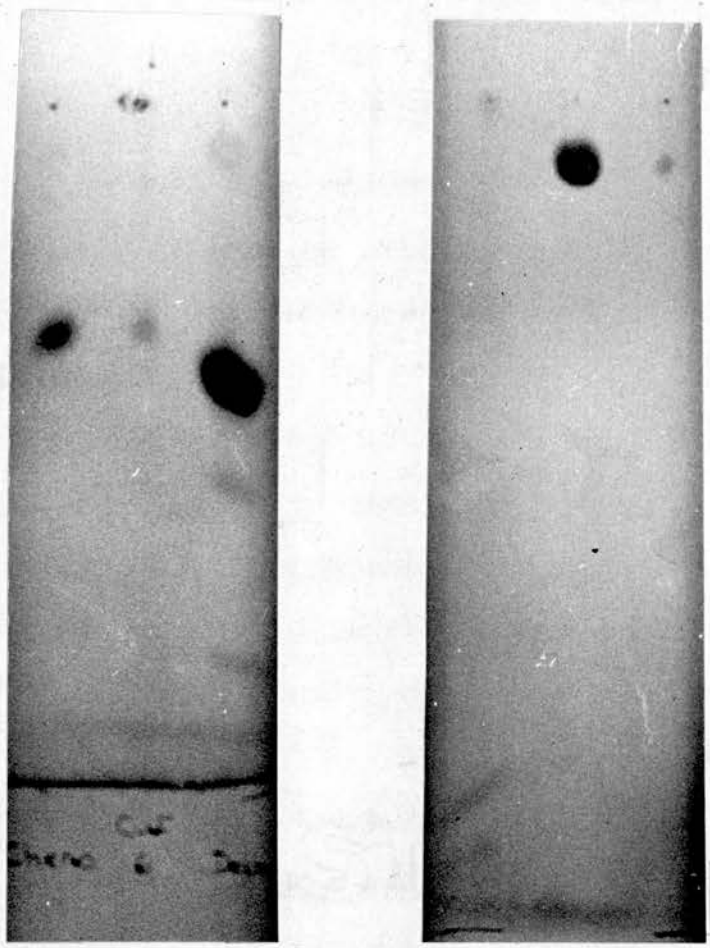
TABLE 10

Experiment (7)

Segment (c/m)	Origin	7α-hydroxy cholesterol	7-keto-cholesterol	cholesterol	cholesterol esters	Ratio ($\frac{\text{choles.}}{\text{esters}}$)	Ratio ($\frac{\text{cholest.}}{7\alpha\text{-}}$)
zero hours	0	3	0	500	2510	1:5	160:1
24 hours	26	5	56	880	3800	1:4.5	170:1

Bile acid column (one half of each cut counted)

	100% pet. ether		20% benzene: 80% pet-ether		40% benzene: 60: pet.-ether (Dihydroxy bile acids)		60% benzene: 40% pet.-ether		80% benzene 20% pet.-ether		100% benz- (Trihydroxy-bile acids)			
Cut No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
c/m	2850	26	24	0	10	100	30	10	27	15	0	30	0	6
Thin layer chromatography						chenodeoxy cholic acid			substance less polar than cholic acid, more polar than chenodeoxy-cholic acid			cholic acid		



1 2 3 4 5 6

Fig. 11

Incubation of C^{14}-cholesterol (and esters) with macrophages. Photographs of thin layer plates obtained by running cuts from the "bile acid column".

1. Standard chenodeoxycholic acid
2. Cut 6
3. Standard deoxycholic acid
4. Cut 9
5. Standard cholic acid
6. Cut 12

acid cut, cut 6, in the trihydroxy-acid cut, cut 12, and in cut 9. Cut 6 was run on thin layer chromatography in trimethyl pentane (10):ethyl acetate (10): and acetic acid (2), together with standard chenodeoxycholic acid and deoxycholic acid. Cut 6 was found to contain a substance which ran with the same mobility as chenodeoxycholic acid (Fig. 11). Cut 9 and cut 12 were run with cholic acid in the same system and cut 12 contained a substance with the same polarity as cholic acid, whereas cut 9 contained a substance with a mobility slightly less than that of cholic acid, but more polar than chenodeoxycholic or deoxycholic acids.

Thus, it would seem that the cholesterol or the esters added to the macrophages in this experiment has indeed been converted into bile acids. It is possible that bile acids, also labelled, could have been present in the original serum, but the zero hour incubation showed no radioactivity in the section of the plate in which bile acids would have run (i.e. the origin segment).

SUMMARY OF SECTION III

A. A preliminary experiment was carried out on the metabolism of C^{14}-labelled cholesterol in the different cell fractions of rat liver, using a boiled tissue incubation as a control for each incubation. It was shown that very little, if any, enzymic metabolism occurred, whereas auto-oxidation products were formed.

B. Preliminary experiments, using cholest-5-en-3β-ol-7α-hydroperoxide as a substrate were carried out. This substance was found to be rather unstable, however, forming both 7α-hydroxycholesterol and 7-ketocholesterol when incubated with buffer alone. The hydroperoxide is unstable, also, when eluted from an alumina column and to a lesser extent from a silicic acid column. However, by using control incubations, it was shown that an active acetone powder of mitochondria could convert the 7α-hydroperoxide of cholesterol to 7α-hydroxycholesterol.

C. Experiments were carried out in which C^{14}-labelled cholesterol and cholesterol esters in rat serum were incubated with growing rat macrophages. After varying time intervals the products of these incubations were examined. It was found that cholesterol or its esters had been metabolised in some incubations to 7α-hydroxycholesterol and more polar products. The more polar products were identified as bile acids. Other incubations seemed quite inactive, autoxidation products only being formed.

SECTION IV

METABOLISM OF 7α-HYDROXYCHOLESTEROL IN RAT LIVER CELL FRACTIONS

METABOLISM OF 7α-HYDROXYCHOLESTEROL

7α-hydroxycholesterol, both labelled with tritium and unlabelled, was incubated with the different cell fractions of rat liver using the methods described above. The metabolism of this substrate in each cell fraction will be discussed separately.

1. MICROSOMES and SUPERNATANT

This is the fraction left after removal of the mitochondria by spinning at 10,000 x g for 15 min. and was studied initially before separating the fraction further into microsomes and supernatant.

(a) Using the incubation mixture and methods of extraction and estimation described, the disappearance of 7α-hydroxycholesterol from incubations with microsomes and supernatant was measured in fifteen experiments. In each case the disappearance of 7α-hydroxycholesterol from "active" tissue was compared with that from boiled tissue and for these fifteen experiments was found to be 30% ± 6%. Using tritiated 7α-hydroxycholesterol as substrate and estimating its disappearance

Graph of NAD concentration plotted against 7α-hydroxycholesterol disappearing

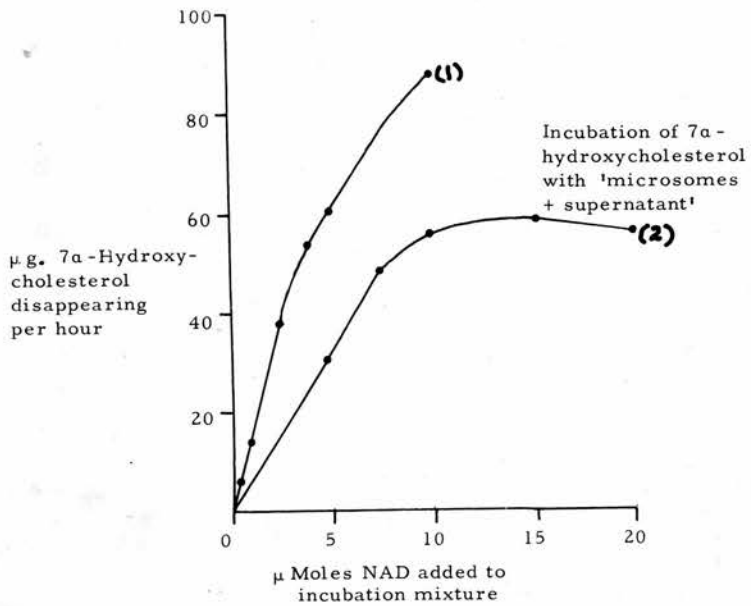


Fig. 12

after incubation by eluting the substrate from a fluorescent thin layer plate confirmed this approximate figure.

(b) This disappearance of 7α-hydroxycholesterol from an incubation mixture was used as a measure of its metabolism (i.e., enzymic activity). Experiments were carried out with eight incubations each containing a different amount of NAD -varying from 0.5 μ mole to 10 μ moles in the incubation mixture in experiment (1) (see Table 11), and from 5 μ moles to 20 μ moles in experiment (2). Boiled tissue controls were included. As can be seen from Table 11 and the corresponding graphs (Fig. 12) the amount of 7α-hydroxycholesterol disappearing per hr. increases as the NAD concentration increases up to a concentration of about 10 μ moles NAD/6 ml. incubation mixture. NAD concentrations of 15 μ moles and 20 μ moles per 6 ml. gave no further increase in 7α-hydroxhcholesterol disappearance. From these experiments it can be concluded that the disappearance of 7α-hydroxycholesterol from incubations with microsomes and supernatant is dependent on the concentration of NAD present up to a certain limiting value, where presumably the enzyme or enzymes become saturated.

TABLE 11

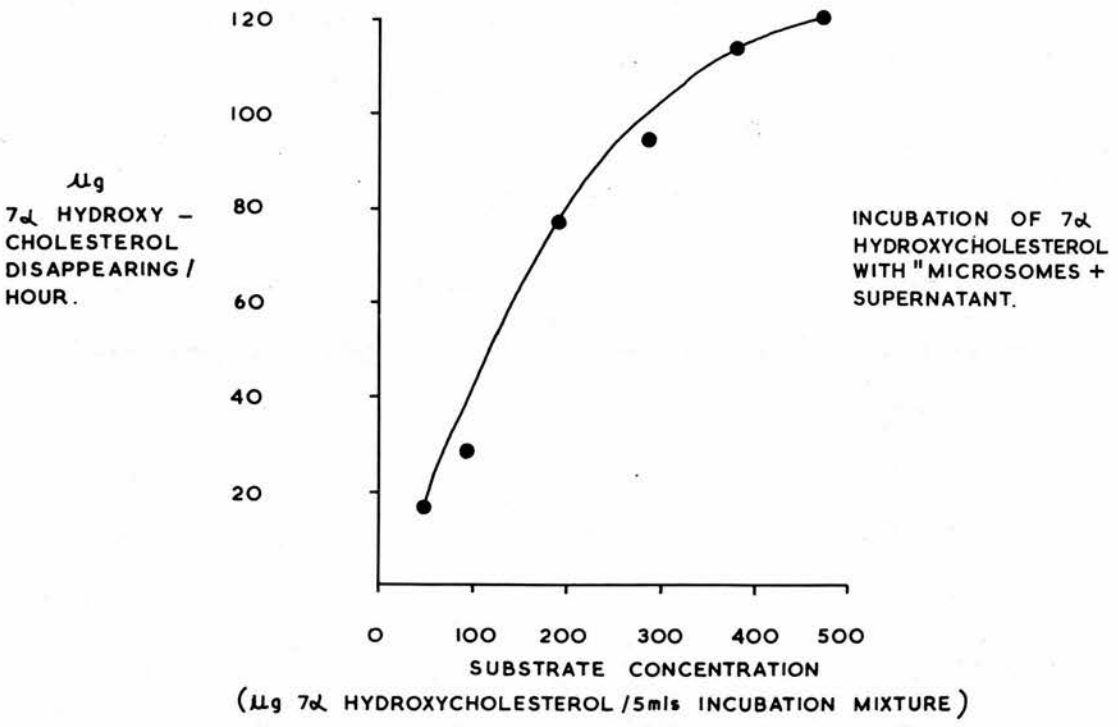
Microsomes and Supernatant

(Variation in NAD concentration
(7α-hydroxycholesterol disappearing measured.

NAD concentration (μ moles/6 ml.)	Experiment (1)	Experiment (2)
	μg. 7α-hydroxycholesterol disappearing/hr.	
0.5	6	
1.0	14	
2.5	38	
4.0	52	
5.0	60	30, 32
7.5		48
10.0	88	56
15.0		58
20.0		56
10.0 Boiled tissue control	8	10
		10

In experiment (1), the values of 7α-hydroxycholesterol disappearing are unusually high.

GRAPH OF SUBSTRATE CONCENTRATION PLOTTED AGAINST REACTION VELOCITY.



Graph of $\frac{1}{S}$ against $\frac{1}{v}$ (incubation of 7α-hydroxy-cholesterol with 'microsomes + supernatant')

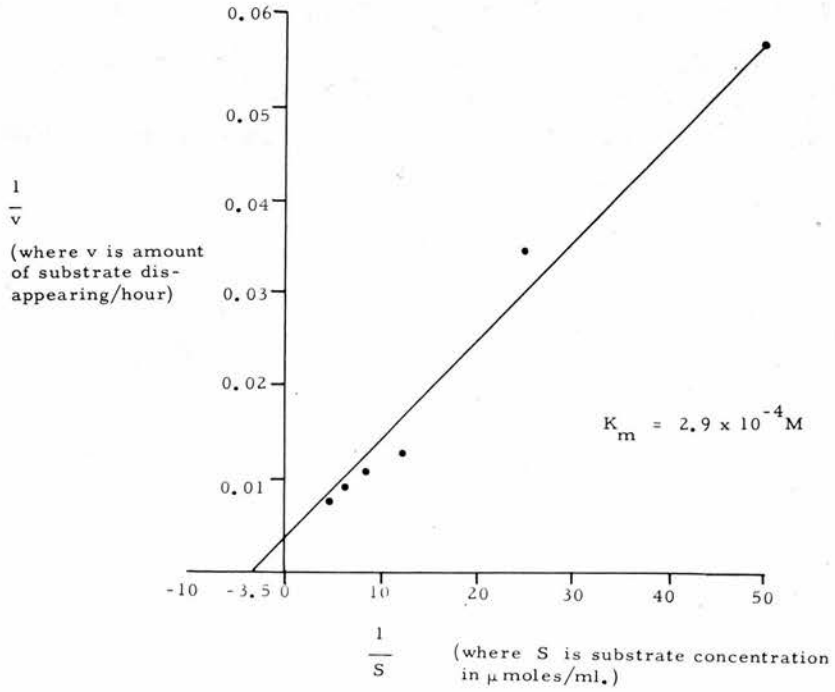


Figure 13

This would indicate that possibly 7α-hydroxycholesterol is being metabolised in this cell fraction to a product containing two hydrogen atoms less than 7α-hydroxycholesterol. Another explanation is that a complicated series of reactions is taking place, and cnly one of these may require NAD as co-factor.

(c) Experiments were carried out in which incubations were set up containing varying amounts of the substrate 7α-hydroxycholesterol. The amount of substrate added varied from 47.5 µg. to 475 µg. and each amount was added in 0.5 ml. methanol. The disappearance of substrate per hr. was measured and plotted against the substrate added (Fig. 13 Table 12). As can be seen as the substrate concentration increased the amount of 7α-hydroxycholesterol disappearing per hr. also increased, giving a curve increasing sharply initially and then flattening off. A graph of the reciprocal of the substrate concentration (I/(s)) against the reciprocal of the initial velocity of reaction (1/v) i.e., the disappearance of 7α-hydroxycholesterol per hr.) was drawn and found to be a straight line giving a K_m value for the reaction of 2.9×10^{-4} M.

TABLE 12

Microsomes and Supernatant

(Variation in substrate concentration
(7α-hydroxycholesterol disappearing
measured.

µg. substrate added in 0.5 ml. methanol		µg. substrate disappearing per hr.
47.5		17.5
95		29
190		78
285		95
380		114
475		135
190	Boiled tissue controls	10
190		10

TABLE 12

Substrate added µ moles/ml.	$\frac{1}{\text{substrate conc(s)}}$	$\frac{1}{\text{reaction velocity (v)}}$
0.02	50	0.057
0.04	25	0.035
0.08	12.5	0.013
0.12	8.3	0.011
0.16	6.25	0.009
0.20	5.0	0.007

(where v, the reaction velocity is equal to the amount of substrate disappearing/hr. in µg./hr.)

TABLE 13

Microsomes + Supernatant

Effect of methanol on enzymic activity

(Variation in substrate concentration
(7α-hydroxycholesterol disappearing measured

µg. substrate added	µg. substrate disappearing when substrate added in 0.1 ml. methanol	µg. substrate disappearing when substrate added in 0.5 ml. methanol
47.5	7.5	9.5
95	29	25
142.5	56.5	52.5
190	70	56
190 Boiled tissue control	10	10

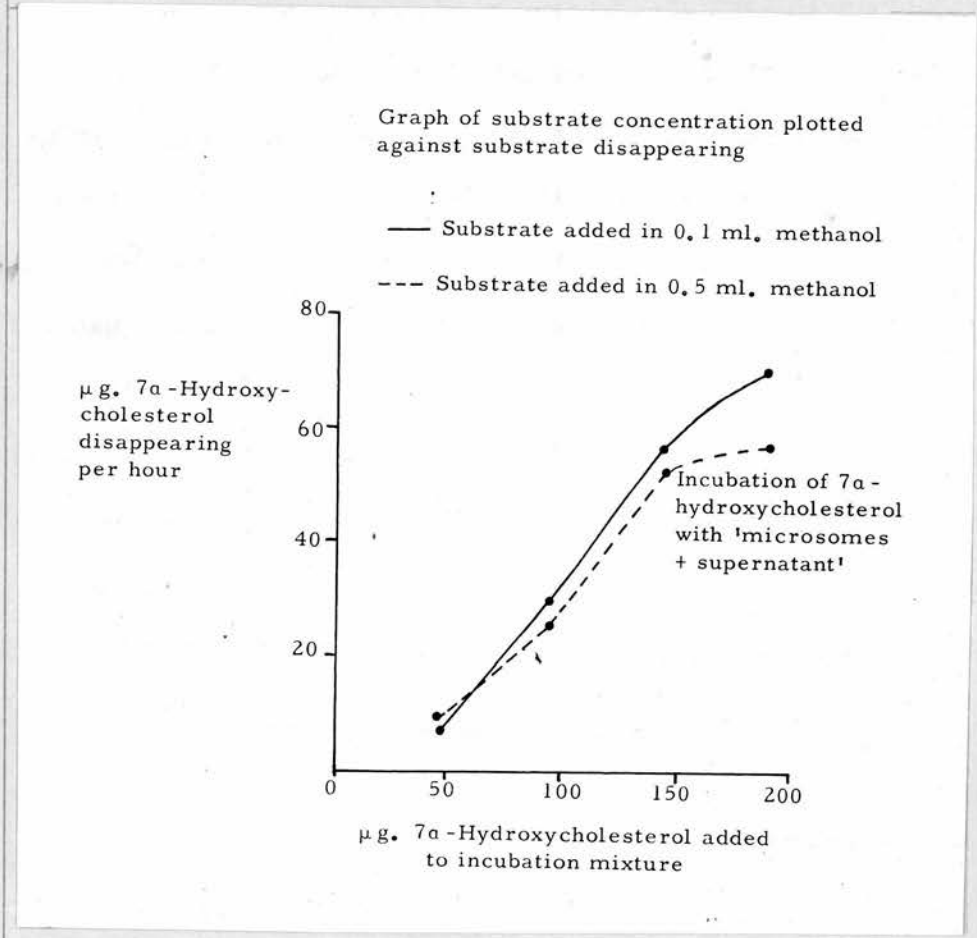

Graph of substrate concentration plotted against substrate disappearing
Substrate added in 0.1 ml. methanol
Substrate added in 0.5 ml. methanol
μg. 7α-Hydroxy-cholesterol disappearing per hour
80
60
40
20
0
50
100
150
200
Incubation of 7α-hydroxycholesterol with 'microsomes + supernatant'
μg. 7α-Hydroxycholesterol added to incubation mixture

Figure 14

In order to show that such large amounts of methanol as 0.5 ml. had no effect on the enzymic activity two experiments using microsomes and supernatant from one rat were carried out, (Tab.13). In one, substrate varying from 50 µg. to 200 µg. in 0.1 ml. methanol was added to the incubation mixtures and the disappearance of substrate compared with that in the second experiment where each amount of substrate was added in 0.5 ml. methanol. Plotting the disappearance of 7α-hydroxycholesterol against substrate concentration in each experiment gave curves (Fig. 14) which hardly differed and it must be concluded that the enzyme or enzymes are unaffected by the 0.5 ml. methanol (nearly 10% of the total incubation mixture).

2. MICROSOMES

a) An attempt was made to measure the disappearance of 7α-hydroxycholesterol from incubations with this fraction by direct estimation of the 7α-hydroxycholesterol in the ether:alcohol cut as described on p. 51. This was found to be impossible, however, due to the fact that the Liftschütz colour obtained was not a typical one but tended to be brownish instead of blue-green.

As the estimation of 7α-hydroxycholesterol in lipid extracts from the microsomes and supernatant fraction was perfectly satisfactory, it must be assumed that some interfering substance exists in microsomes alone, which, as it does not occur in microsomes and supernatant must either be metabolised or removed in some way when supernatant is added to the microsomes. Therefore the only way in which 7α-hydroxycholesterol could be estimated after incubation with microsomes was to run the concentrated ether:alcohol cut from the alumina column on the thin layer plate to separate it from interfering substances and then to elute it as described on p. 60 . In this way it was found that when 7α-hydroxycholesterol was incubated with microsomes and NAD (10 μ moles) approximately 30% of it disappeared. When no NAD was added the disappearance was only about 4%.

(b) Isolation of metabolites

Using the technique of thin layer chromatography on fluorescent plates described on p. 56 and comparing the ether:alcohol cuts from the alumina columns from an incubation involving boiled tissue with one involving active tissue, showed

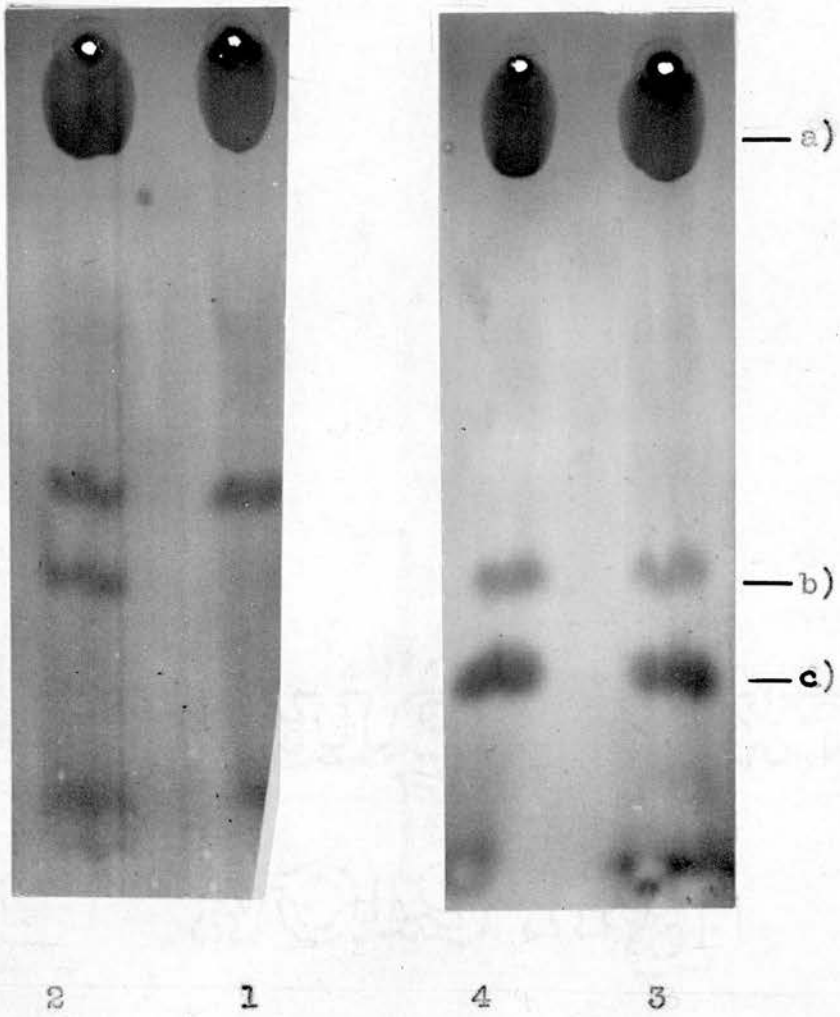


Fig. 15

Photographs of fluorescent plates, illuminated by u.v. light.

Plates obtained from incubations of microsomes with 7α-hydroxycholesterol, with varying NAD concentration

1. No NAD added
2. 5 μ moles NAD
3. 10 μ moles NAD
4. 15 μ moles NAD

u.v. absorbing substances:-

a) nicotinamide
b) 7-ketocholesterol
c) cholest-4-en-3-one-7α-ol

Ultraviolet absorption spectra of cholest-4-en-3-one-7α-ol and the product obtained from incubations with 7α-hydroxy-cholesterol

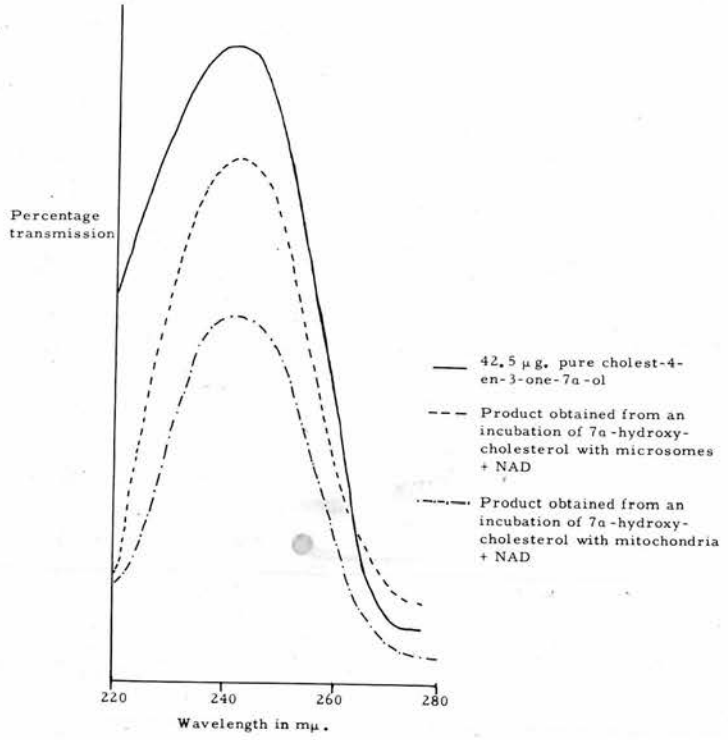


Fig. 16

that in the presence of active tissue and NAD, a u.v. absorbing substance was formed, (Fig. 15). When labelled 7α-hydroxycholesterol was used as substrate, this metabolite was also found to be labelled, indicating that it must have been produced from 7α-hydroxycholesterol. It seemed possible that the metabolite might be cholest-4-en-3-one-7α-ol, and the metabolite actually had the same R_F value in the benzene:ethyl acetate:acetone (10:5:3) system as an authentic sample of cholest-4-en-3-one-7α-ol[1] (i.e., 0.66), and also in the benzene:dioxane (9:1) system (i.e., 0.10).

When the metabolite was isolated from a thin layer plate, it was found to have the same absorption spectrum as cholest-4-en-3-one-7α-ol (Fig. 16) having a peak at 242 mμ., indicating the presence of an α,β-unsaturated ketone. The substance also gave a pink colour with phosphotungstic acid when sprayed on a plate, as does cholest-4-en-3-one-7α-ol (see specificity of phosphotungstic acid test, Appendix, p. 310).

When the substance produced from an incubation with labelled substrate was mixed with unlabelled cholest-4-en-3-one-7α-ol and co-chromatography was carried out in the benzene:ethyl

[1] The preparation of this substance is described in the Appendix, p. 304 .

acetate:acetone system and in the benzene:dioxane system the substance produced from 7α-hydroxycholesterol did not separate in either system from cholest-4-en-3-one-7α-ol. When the plates were segmented into five sections, eluted, and counted, the radioactivity was found to be concentrated in this one u.v. absorbing spot.

Labelled metabolite was isolated from several incubations, pooled, and mixed with 5 mg. unlabelled cholest-4-en-3-one-7α-ol. The mixture was crystallised three times from methanol until constant specific activity was achieved. A sample from each crystallisation was taken, its absorption at 242 mμ. read, and then counted to obtain the specific activity of the sample (Table 14). An infra-red spectrum of the final sample was plotted, and this compared well with an infra-red spectrum of authentic cholest-4-en-3-one-7α-ol.

The cumulative evidence produced here indicates then, that in microsomes 7α-hydroxycholesterol is converted into cholest-4-en-3-one-7α-ol in the presence of NAD.

TABLE 14

Microsomal Enzyme

Cholest-4-en-3-one-7αol formed by incubation of 7α-hydroxycholesterol (labelled) with enzyme and NAD, crystallised to constant specific activity with 5 mg. of the authentic compound.

	1st crystallisation	2nd crystallisation	3rd crystallisation
Optical density at 242 mμ.	0.843	0.908	0.825
μg. present in sample	66	71	65
c/m present in sample	580	680	626
∴ c/m/μg.	8.8	9.5	9.5
c/m/mg.	8800	9500	9500

Using the fluorescent thin layer chromatography plates as a means of identifying metabolites produced from 7α-hydroxycholesterol, no evidence has been found from an examination of any of the cuts of the alumina columns to suggest that any product other than cholest-4-en-3-one-7α-ol is formed. For example, no further u.v. absorbing substances which are formed only in "active" incubations have ever been seen. 7-ketocholesterol is indeed formed in all incubations with active or boiled tissue, but as the same amount, approximately, is formed in every case (estimated by absorption at 238 mμ. or by radioactive measurements) it must be concluded that 7-ketocholesterol is formed from 7α-hydroxycholesterol only by auto-oxidation. 7β-hydroxycholesterol is also seen to be formed auto-oxidatively.

Spraying plates obtained from incubations with active tissue with phosphotungstic acid or with phosphomolybdic acid showed up no additional substances which did not occur on plates obtained from boiled control incubations. When the plates obtained from incubations with active and boiled tissue were segmented, eluted and counted, it was seen that the only radioactive substances formed

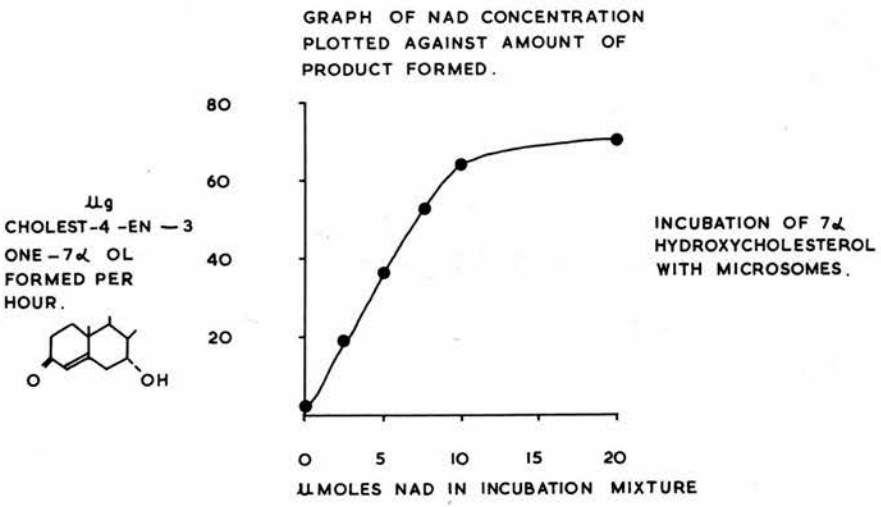
in the active incubations were cholest-4-en-3-one-7α-ol and 7-ketocholesterol, whereas in the boiled control incubations the latter substance alone was produced.

When no NAD was added to incubations with microsomes and 7α-hydroxycholesterol, only a trace of cholest-4-en-3-one-7α-ol could be detected, and no other products were found. As has been mentioned on p.105 the disappearance of 7α-hydroxycholesterol amounted only to about 4%.

(c) It is now possible to estimate not only the disappearance of 7α-hydroxycholesterol from an incubation but also the appearance of cholest-4-en-3-one-7α-ol under various conditions. It was measured, as described above (p. 60) by marking its position on the fluorescent thin layer plate, scraping off this area, and extracting the silicic acid with ether:alcohol. This extract was taken to dryness, dissolved in methanol, and the absorption of the solution read at 242 mμ.

(i) Effect of varying the NAD concentration

A series of incubations was set up, each containing the same amount of substrate, but the NAD concentration was varied from zero up to 20 μ moles.


GRAPH OF NAD CONCENTRATION PLOTTED AGAINST AMOUNT OF PRODUCT FORMED.
μg CHOLEST-4-EN—3 ONE-7α OL FORMED PER HOUR.
80
60
40
20
O
OH
0 5 10 15 20
μMOLES NAD IN INCUBATION MIXTURE
INCUBATION OF 7α HYDROXYCHOLESTEROL WITH MICROSOMES.

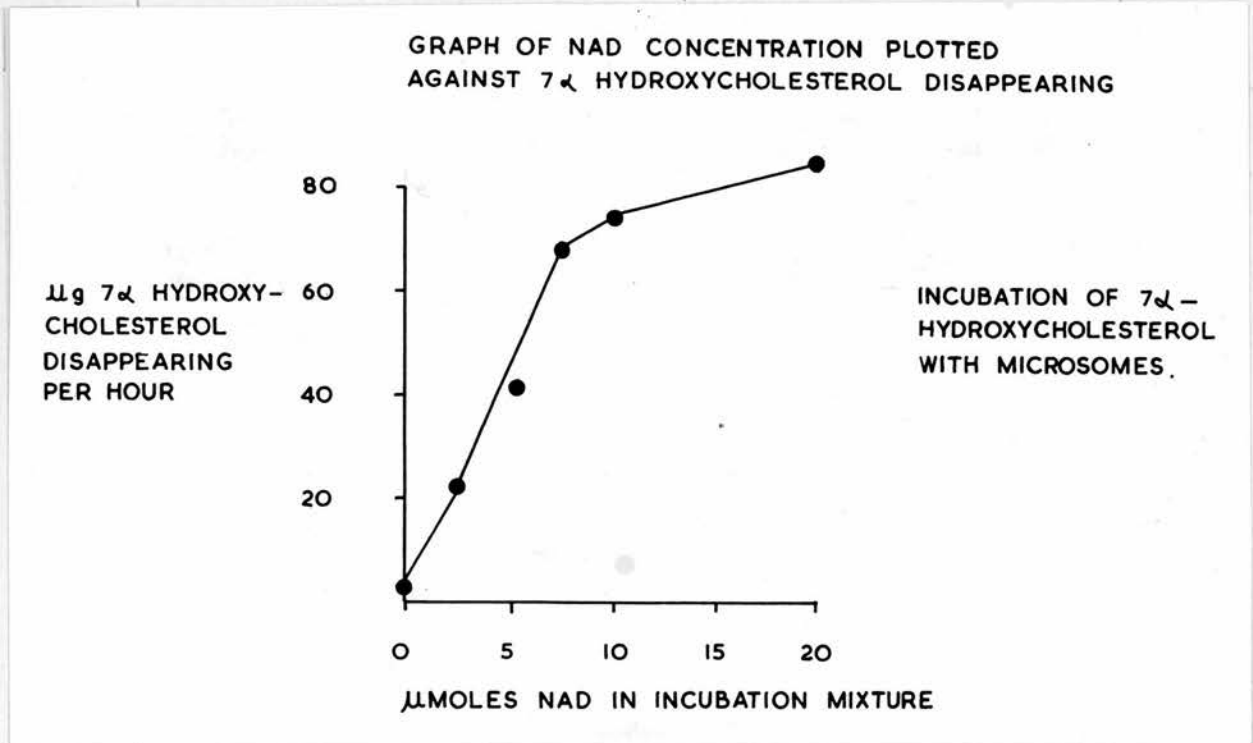

GRAPH OF NAD CONCENTRATION PLOTTED AGAINST 7α HYDROXYCHOLESTEROL DISAPPEARING
μg 7α HYDROXY-CHOLESTEROL DISAPPEARING PER HOUR
80
60
40
20
0 5 10 15 20
μMOLES NAD IN INCUBATION MIXTURE
INCUBATION OF 7α-HYDROXYCHOLESTEROL WITH MICROSOMES.

Fig. 17

In each incubation both the amount of cholest-4-en-3-one-7α-ol formed and also the amount of 7α-hydroxycholesterol disappearing was estimated. From the Tables below and Fig. 17, it can be seen that, up to about 10 μ moles of NAD in the incubation mixture, the amount of cholest-4-en-3-one-7α-ol increased proportionately with the disappearance of 7α-hydroxycholesterol. At 20 μ moles of NAD no further increase in the amount of cholest-4-en-3-one-7α-ol formed was observed, and the disappearance of 7α-hydroxycholesterol corresponded with this.

Microsomes

Variation in NAD concentration
(7α-hydroxycholesterol disappearing measured
(Cholest-4-en-3-one-7α-ol appearing measured

NAD conc. μmoles/6 ml.	μg. 7α-hydroxy-cholesterol disappearing	μg. cholest-4-en-3-one-7α-ol appearing
0	5	3
2.5	22	19
5	41	40
7.5	70	53
10	77	64
20	84	70

It can therefore be concluded that 7α-hydroxycholesterol is converted into cholest-4-en-3-one-7α-ol in microsomes and that this conversion is dependent upon NAD as co-factor. At levels of about 10 μ moles of added NAD the enzyme must be saturated, and at this level about 30% of the added 7α-hydroxycholesterol has been converted to this product. Again it must be assumed that large amounts of added NAD are required because of the other NAD-requiring reactions occurring in microsomes.

A further experiment showed that NADP could replace NAD as co-factor.

(ii) Effect of varying the substrate concentration

Six incubations were set up, each containing microsomes and 10 μ moles NAD and a different amount of substrate, ranging from 60 μg. to 600 μg. each amount being added in 0.25 ml. methanol.

The ether-alcohol cuts obtained from these incubations were run on fluorescent thin layer plates and the cholest-4-en-3-one-7α-ol formed in each case was eluted and estimated by its absorption at 242 mμ. The 7α-hydroxycholesterol recovered in each case was also eluted and estimated by the Lifschütz colour reaction.

TABLE 15

MICROSOMES

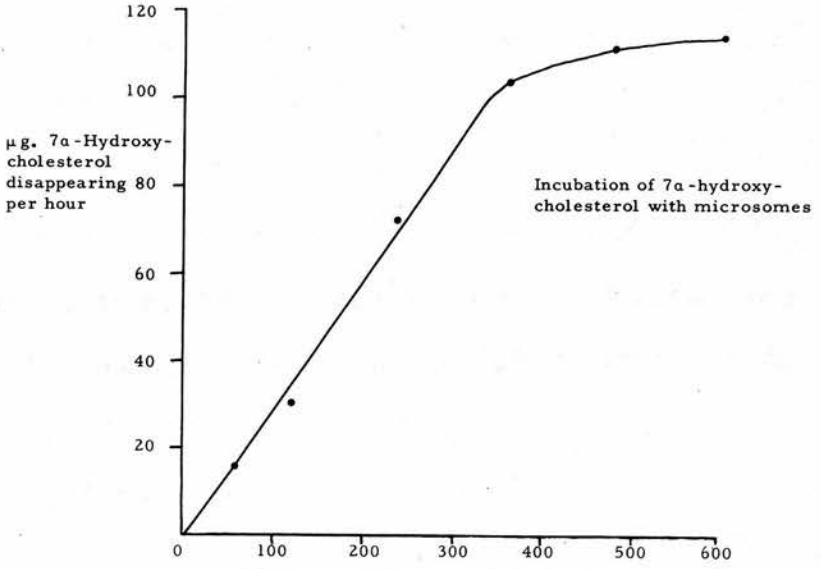
Variation in substrate concentration

(7α-hydroxycholesterol disappearing measured
)cholest-4-en-3-one-7α-ol formed measured

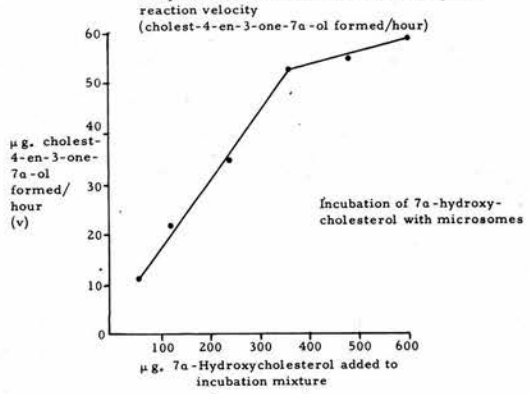
7α-hydroxycholesterol added to incubation μg.	Cholest-4-en-3-one-7α-ol formed per hr. μg.	7α-hydroxycholesterol disappearing per hr. μg.
60	11	15
120	21	30
240	34	72
360	52	104
480	54	112
600	59	114
Substrate added μ Moles/ml (s)	$\frac{1}{s}$	$\frac{1}{v}$
0.025	40	0.091
0.05	20	0.048
0.1	10	0.029
0.15	6.66	0.019
0.20	5.0	0.0185
0.25	4.0	0.0169

(where v = reaction velocity, i.e. the amount of cholest-4-en-3-one-7α-ol formed/hr. in μg./hr.

Graph of substrate concentration plotted against substrate disappearing per hour



Graph of substrate concentration plotted against reaction velocity
(cholest-4-en-3-one-7α-ol formed/hour)



Graph of $\frac{1}{S}$ against $\frac{1}{v}$
(incubation of 7α-hydroxycholesterol with microsomes)

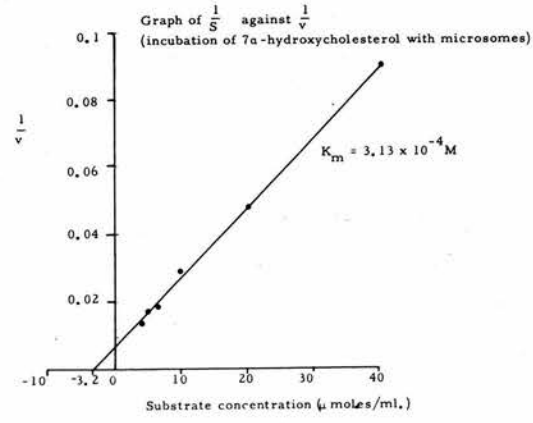


Figure 18

Table 15 and the graphs of the results (Fig.18 are shown opposite. The amount of cholest-4-en-3-one-7α-ol formed was plotted against substrate concentration and then a graph was drawn of the reciprocal of the substrate concentration against the reciprocal of the initial reaction velocity (i.e., the amount of cholest-4-en-3-one-7α-ol formed per hr). The amount of 7α-hydroxycholesterol disappearing per hr. was also plotted against substrate concentration.

From the graph it can be seen that the amount of cholest-4-en-3-one-7α-ol increases as the substrate concentration increases giving a curve typical of an enzymic reaction. The graph of $\frac{1}{v}$ against $(\frac{1}{s})$ was found to be a straight line giving a K_m value for the reaction of 5.13×10^{-4} M.

The disappearance of 7α-hydroxycholesterol was also shown to increase with substrate concentration.

Ref to p. 102

(iii) Effect of cyanide on the oxidation of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol.

Four incubations were set up, with labelled 7α-hydroxycholesterol with the compositions

(a) microsomes + 10 μ moles NAD

(b) microsomes + 10 μ moles NAD + 10 μ moles potassium cyanide

Graph of cyanide added plotted against product formed

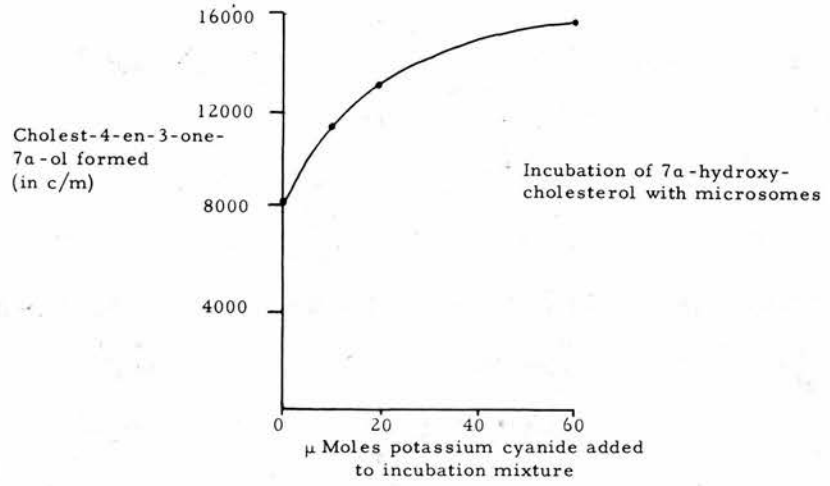


Fig. 19

Graph of pH plotted against product formed

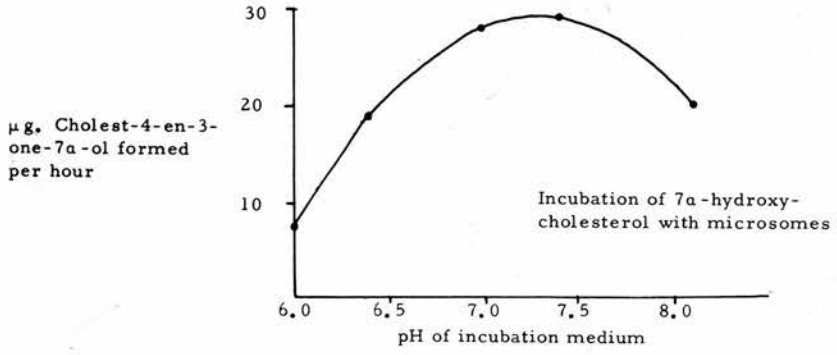


Fig. 20

(c) microsomes + 10 μ moles NAD + 20 μ moles potassium cyanide

(d) microsomes + 10 μ moles NAD + 60 μ moles potassium cyanide.

In each incubation the cholest-4-en-3-one-7α-ol produced was isolated on fluorescent thin layer plates, eluted and estimated by the absorption at 242 mμ. and also by radioactive methods. The amount of cholest-4-en-3-one-7α-ol produced was plotted against the amount of cyanide in the incubation mixture and the curve shown in Fig. 19 was obtained.

Incubation	Cholest-4-en-3-one-7α-ol produced(c/m)
(a) no KCN	8270
(b) 10 μ moles KCN	11,560
(c) 20 μ moles KCN	13,210
(c) 60 μ moles KCN	15,850

(140 μg. substrate added = 70,000 c/m; specific activity = 500 c/m/μg.).

The results show that, contrary to expectation the amount of cholest-4-en-3-one-7α-ol produced increases as the cyanide concentration in the incubation mixture increases. Thus cyanide does/ not

inhibit the reaction but actually "activates" the enzyme or enzymes. At a concentration of 60 μ moles of cyanide the amount of cholest-4-en-3-one-7α-ol produced is nearly double that formed when no cyanide is present.

The behaviour of the cyanide could be explained if it is inhibiting the other NAD-requiring reactions occurring in the microsomes, thus leaving more NAD available to act as co-factor in the oxidation of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol.

(iv) Effect of changes in pH on the oxidation of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol

Five incubations were set up each with microsomes, 10 μ moles NAD and 7α-hydroxycholesterol. The pH was varied from 6.0 to 8.1 by the steps 6.0, 6.4, 7.0, 7.4 and 8.1 by incubating with phosphate buffer of the appropriate pH. The cholest-4-en-3-one-7α-ol produced in each case was isolated from the thin layer plates, and estimated by its absorption at 242 mμ.

The amount of cholest-4-en-3-one-7α-ol formed was plotted against pH, and the graph shown in Fig. 20 was obtained.

pH of incubation mixture	Cholest-4-en-3-one-7α-ol produced (μg.)
6.0	7.5
6.4	19
7.0	28
7.4	29
8.1	20

The results indicate that the microsomal enzyme has its highest activity when incubated in a medium of pH between 7.0 and 7.4, i.e. the pH optimum of the enzyme is between 7.0 and 7.4.

(v) Effect of succinate on the microsomal oxidation of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol

In later experiments, using the mitochondrial fraction of rat liver, it was found that succinate had an effect on the metabolism of 7α-hydroxycholesterol, presumably by reducing the added NAD to NADH (p. 144). It was therefore decided to incubate succinate with microsomes, 10 μ moles NAD and labelled 7α-hydroxycholesterol to discover if succinate had the same effect in microsomes as it has in mitochondrial incubations.

Incubations were set up as shown in Table 16 and the cholest-4-en-3-one-7α-ol formed in each

case was isolated and estimated both by determining its absorption at 242 mμ. and by radioactive means. The results shown in the Table indicate that succinate has no marked effect on the production of cholest-4-en-3-one-7α-ol. The incubation in which 72 μ moles of succinate were used did, in fact, seem to give slightly less

TABLE 16

Incubation	Cholest-4-en-3-one-7α-ol formed (c/m)
a) Microsomes + NAD	5060
b) Microsomes + NAD + 36 μ moles succinate	5240
c) Microsomes + NAD + 72 μ moles succinate	4500

cholest-4-en-3-one-7α-ol, but the difference between the three results is hardly significant. In mitochondria, on the other hand, the presence of even 7.2 μ moles of succinate with 10 μ moles of NAD gave rise to about one-thirtieth of the cholest-4-en-3-one-7α-ol produced by mitochondria with NAD and no succinate. Thus, if succinate has an effect on the production of cholest-4-en-3-one-7α-ol in microsomes it is a very small one indeed compared to the effect occurring in incubations with mitochondria.

(vi) Incubations of 7α-hydroxycholesterol with an acetone powder of microsomes

An acetone powder of microsomes was prepared as described on p. 39 . The powder was dissolved in buffer solution and incubated with 7α-hydroxycholesterol and NAD. By viewing the fluorescent thin layer plates it was seen that cholest-4-en-3-one-7α-ol had been formed. The amount of this substance formed increased as the amount of acetone powder was increased, the cholest-4-en-3-one-7α-ol being estimated by radioactive measurements.

Thus it seems that the oxidation reaction occurring in microsomes when cholest-4-en-3-one-7α-ol is formed from 7α-hydroxycholesterol is not affected when the microsomes are treated in this way.

3. SUPERNATANT

This is the cell fraction left after the microsomes have been sedimented by spinning at 100,000 x g for 60 min. This fraction is a clear red solution.

(a) The disappearance of 7α-hydroxycholesterol from incubations with the supernatant fraction fortified with NAD has only been measured in five experiments. With this fraction, as with microsomes and supernatant, the 7α-hydroxycholesterol can be estimated directly in the ether:alcohol cuts from the alumina columns as there seem to be no interfering substances, as in microsomes. The disappearance of 7α-hydroxycholesterol was found to be about 10% in these experiments.

In experiments using labelled 7α-hydroxycholesterol the disappearance was confirmed to be 10%

(b) Identification of metabolites

(i) On running the ether:alcohol cuts from the alumina columns from incubation mixtures of "boiled" and "active" supernatant with NAD added it was seen that in the "active" incubation a u.v. absorbing substance had been formed. This substance had the same characteristics as cholest-4-en-3-one-7α-ol, i.e., the R_F value in the benzene: ethyl acetate:acetone thin layer system was 0.66 and it gave an absorption spectrum with a peak at 242 mμ. By using labelled substrate it was shown

that this substance was also radioactive, indicating that it had been formed from 7α-hydroxycholesterol. Estimating this product both by radioactive measurements and by its absorption at 242 mμ. showed a very small amount (2%) had in fact been formed from 7α-hydroxycholesterol.

7-ketocholesterol was shown to be formed in incubations with both boiled and active tissue, and again only 2% was produced. Incubations carried out with no added NAD showed that no cholest-4-en-3-one-7α-ol was formed.

(ii) On running the ether cuts from the alumina columns obtained from incubations with boiled and active tissue it was seen that a substance which gave a blue colour on spraying the plate with phosphotungstic acid was formed in the incubation with active tissue. This substance was fairly non-polar as it was eluted in the ether cut from alumina and from its mobility on the plate in the benzene:dioxane (19:1) system, was thought to be an ester of 7α-hydroxycholesterol. The R_F values of some synthetic 7α-hydroxycholesterol esters (prepared by Dr. Mawer, 1962) in this chromato-

TABLE 17

Steroid	R_F Values in Benzene:Dioxane (19:1)
7α-Hydroxycholesterol	0
7α-hydroxycholesterol	
- acetate	0.24
- laurate	0.38
- myristate	0.38
- stearate	0.63
Ester formed on incubation	0.40

graphic system are shown in Table 17 and compared with the R_F value of this substance produced in supernatant incubations. The synthetic esters are eluted from alumina also in the ether cut.

When labelled 7α-hydroxycholesterol was used as substrate it was shown that this substance was also labelled and that about 3% was formed from 7α-hydroxycholesterol in "active" incubations.

(iii) A complete radioactive analysis of the extracts from incubations was carried out by running the three cuts from the alumina columns on thin layer plates which were segmented and counted. This showed that no further metabolites were formed in incubations of 7α-hydroxycholesterol with active supernatant, either with or without added NAD.

4. MITOCHONDRIA

This cell fraction is obtained after removal of nuclei, cell debris etc., by spinning the supernatant at 10,000 x g for 15 min. The mitochondria are always washed with sucrose and re-sedimented.

(a) In this fraction, 7α-hydroxycholesterol can be estimated directly by carring out a Lifschütz test on the contents of the ether:alcohol cut from the alumina column. In the presence of NAD it was found that about 15% of the 7α-hydroxycholesterol disappeared from an "active" incubation. When no NAD was added, however, about 4% disappearance could be detected.

Using tritiated substrate and estimating the disappearance by radioactive measurements confirmed this value of approximately 15% disappearance in the presence of NAD.

(b) Identification of metabolites

(i) Comparing thin layer plates obtained from incubations with "boiled" and "active" tissue with 7α-hydroxycholesterol and added NAD showed that in the "active" incubation a u.v. absorbing substance

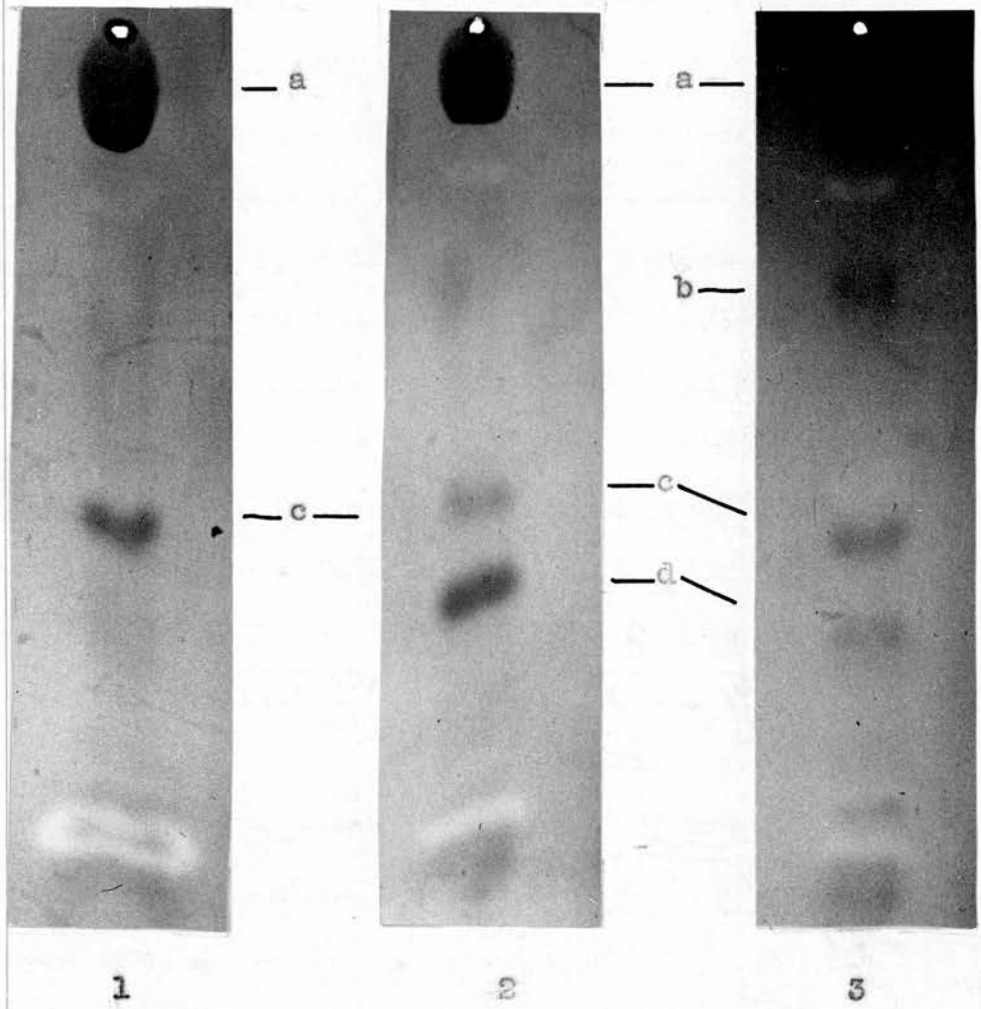


Fig. 21

Photographs of fluorescent thin layer plates, obtained from incubations of 7α-hydroxycholesterol with mitochondria and NAD, illuminated by u.v. light.

1. Boiled mitochondria, and 10 μ moles NAD
2. Mitochondria and 10 μ moles NAD
3. Mitochondria, 10 μ moles NAD and 3 ml. SF

u.v. absorbing substances:

a) nicotinamide
b) "polar u.v. absorbing material"
c) 7-ketocholesterol
d) cholest-4-en-3-one-7α-ol

with a mobility identical with that of cholest-4-en-3-one-7α-ol was formed (see Fig. 21). The substance, when eluted from a plate had an absorption spectrum identical with that of cholest-4-en-3-one-7α-ol (see Fig. 16), and it also gave a pink colour when sprayed with phosphotungstic acid. Incubating labelled 7α-hydroxycholesterol with mitochondria and NAD gave rise to labelled product confirming that it had been formed from the substrate and measurement of the amount formed both by radioactive methods and by reading the absorption at 242 mμ., indicated that about 6% was usually formed. This type of estimation was carried out twelve times.

Thus it would seem that mitochondria with added NAD can also produce cholest-4-en-3-one-7α-ol from 7α-hydroxycholesterol, although, from the same weight of liver the enzyme (or enzymes) is not as active as the enzyme occurring in microsomes. For example, mitochondria equivalent to 1 g. wet weight of liver when incubated with 7α-hydroxy cholesterol and 10 μ moles of NAD gave 7% cholest-4-en-3-one-7α-ol whereas microsomes equivalent to the same amount of liver, when incubated with 7α-hydroxycholesterol and 10 μ moles of NAD gave rise to 25% cholest-4-en-3-one-7α-ol.

(ii) As can be seen from the photograph of the fluorescent thin layer plates obtained from incubations with boiled and active tissue (Fig. 21), 7-ketocholesterol is formed in both cases. This must again be produced by autoxidation and is formed from 7α-hydroxycholesterol, as it is labelled after incubations with labelled substrate. 7β-hydroxycholesterol, in very small quantity was also seen to be formed autoxidatively.

(iii) No other metabolites could be detected in incubations with 7α-hydroxycholesterol and mitochondria with NAD, either from a complete radioactive analysis of the incubation extract, or by spraying the plates and comparing with those obtained from "boiled" control incubations.

(c) Production of cholest-4-en-3-one-7α-ol from 7α-hydroxycholesterol, studied under various conditions

(i) Effect of varying the substrate concentration on the production of cholest-4-en-3-one-7α-ol

Incubations were set up containing mitochondria 20 μ moles NAD and amounts of 7α-hydroxycholesterol varying from 50 μg. to 450 μg. The cholest-4-en-3-one-7α-ol formed in each case was isolated from

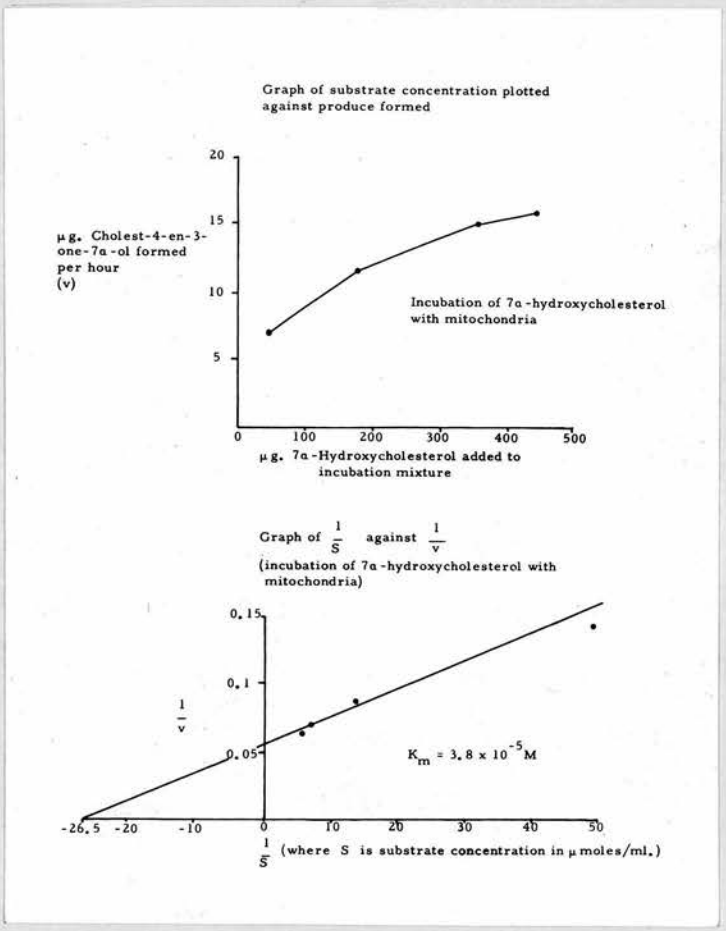

Graph of substrate concentration plotted against produce formed
μg. Cholest-4-en-3-one-7α-ol formed per hour (v)
20
15
10
5
0
100
200
300
400
500
Incubation of 7α-hydroxycholesterol with mitochondria
μg. 7α-Hydroxycholesterol added to incubation mixture
Graph of 1/S against 1/v
(incubation of 7α-hydroxycholesterol with mitochondria)
0.15
0.1
0.05
1/v
K_m = 3.8 x 10^-5 M
-26.5
-20
-10
0
10
20
30
40
50
1/S (where S is substrate concentration in μ moles/ml.)

Figure 22

the thin layer plates obtained from the incubations and estimated by reading the absorption at 242 mμ.

The results are shown in Table 18.

TABLE 18.

Substrate conc. μg./6 ml.	Cholest-4-en-3-one-7α-ol formed. μg./hr.
50	7.0
180	11.4
360	14.6
450	15.7

A graph was drawn of substrate concentration against the amount of cholest-4-en-3-one-7α-ol formed per hr. (Fig. 22), and this showed that the amount of cholest-4-en-3-one-7α-ol increased slowly with increasing substrate concentration. A graph of the reciprocal of the substrate concentration against the reciprocal of the reaction velocity was constructed and found to be a straight line which cut the horizontal axis at such a point as to give a K_m value for the reaction of 3.8×10^{-5} M. This K_m value is quite different from that obtained for the microsomal oxidation of

7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol which would indicate that different enzymes are involved in the two reactions and that the mitochondrial reaction is not due merely to microsomal contamination.

The values calculated for the graph of $\frac{1}{s}$ against $\frac{1}{v}$ are given in Table 19.

TABLE 19

μg. substrate added/ 6 ml.	50	180	360	450
μ moles/ml. substrate added	0.021	0.075	0.15	0.188
1/(substrate conc.)	47.6	13.3	6.66	5.32
1/v (μg./hr.)	0.143	0.087	0.068	0.063

(ii) Effect of varying the NAD concentration on the production of cholest-4-en-3-one-7α-ol

This experiment has been described later in this section (e, ii) in connection with the effect of SF on the metabolism of 7α-hydroxycholesterol in mitochondria. From the tables and graphs given there (p. 140) it can be seen that the amount of cholest-4-en-3-one-7α-ol formed depends on the amount of NAD present in the incubation. The

amount of cholest-4-en-3-one-7α-ol formed increases up to about 20 μ moles and then the curve flattens off slightly up to 40 μ moles. These results show, however, that the enzyme is not saturated at 10 μ moles NAD.

(iii) Effect of cyanide on enzymic activity

Incubations were set up to discover whether cyanide had any effect on the production of cholest-4-en-3-one-7α-ol from 7α-hydroxycholesterol. Labelled 7α-hydroxycholesterol was incubated with mitochondria and 10 μ moles of NAD, and also with mitochondria, 10 μ moles NAD and 10 μ moles of potassium cyanide.

The cholest-4-en-3-one-7α-ol formed in each case was marked on the fluorescent thin layer plates, eluted and estimated both by its absorption at 242 mμ. and by radioactive means.

The results are shown in the Table below:

TABLE 20

Incubation	Cholest-4-en-3-one-7α-ol formed (c/m)	Percentage conversion
a) Mitochondria + NAD	4100	6%
b) Mitochondria + NAD + cyanide	7750	10%

140 μg. substrate added (70,000 c/m)

These results are similar to those obtained with microsomes, and are equally unexpected. It was thought that cyanide would probably inhibit the oxidation reaction, but, in fact, the addition of cyanide increases the percentage conversion of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol from 6% to 10%. This could be explained if the cyanide was inhibiting other reactions occurring in the mitochondria which would normally be using up added NAD.

From the NAD concentration variation experiment (c, ii), it was found that the enzyme was not saturated at 10 μ moles NAD and therefore the explanation for the behaviour of cyanide when 10 μ moles NAD are present is probably valid.

(d) The influence of boiled supernatant fraction (SF) on the metabolism of 7α-hydroxycholesterol.

The preparation of this boiled supernatant fraction (SF) has been described on p. 38. As so much importance has been laid on this SF in cholesterol metabolism (see Introduction, p. 20) when combined with mitochondrial preparations, it was thought to be advantageous to study its effect on the disappearance of 7α-hydroxycholesterol from incubations with mitochondria and NAD.

(i) Effect of SF on disappearance of 7α-hydroxycholesterol

Incubations were carried out in which the disappearance of the unlabelled substrate was estimated by the Lifschütz reaction as has been described (p. 309). Mitochondria, mitochondria + 3 ml. SF, and boiled mitochondria + 3 ml. S.F were compared, all of these containing added NAD. The disappearance of 7α-hydroxycholesterol from boiled mitochondria + SF was about 3 - 5% and from mitochondria, as has been mentioned above (p. 124) was 15%. However the disappearance from active mitochondria + SF increased to 30 ± 5% measured in fifteen experiments. When this increase was discovered it was thought that SF had indeed an effect on the metabolism of 7α-hydroxycholesterol, but, at this stage of the work, no metabolites of any of the cell fractions had been identified, and therefore the way in which SF affected this increase was not known.

In later studies involving radioactive substrate, in which a complete radioactive analysis of the extracts from such incubations was made, no increase in any of the metabolites formed when SF was added could account for this increase in the

disappearance of 7α-hydroxycholesterol. In fact, although nearly the total number of counts added to these incubations could be recovered and accounted for, from boiled mitochondria + SF and from active mitochondria, only 85% of the added counts could be recovered from incubations with active mitochondria + SF. Every vessel, etc., used in the whole procedure was checked for radioactivity and the tissue residue was extracted for 6 hr. in a Soxhlet apparatus with ethyl acetate, and even then 10% of the added counts could not be recovered. The only explanation of this phenomenon is that 10% of the counts, either still as 7α-hydroxycholesterol or as products, must be bound very firmly to the tissue and that SF plays some part in this binding. This "binding" phenomenon is not unknown as Szego (1953), incubating C^{14}-labelled oestrone with rat serum, could only recover the total counts added, by strong acid hydrolysis of the protein. Szego and Roberts (1956) showed that addition of rat liver homogenate to the serum increased the binding and attributed this effect to an enzymic mechanism in the liver tissue, capable of promoting the binding of labelled oestrone, oestradiol and their metabolites to the rat serum proteins.

Thus, as will be seen in the following pages, the increase in disappearance of 7α-hydroxycholesterol when SF is added to mitochondria is not, as had been previously thought, due to an increase in the formation of any metabolite of 7α-hydroxycholesterol.

(ii) Effect of SF on the metabolites formed from 7α-hydroxycholesterol

When mitochondria, fortified with 10 μ moles of NAD are incubated with 7α-hydroxycholesterol, about 6% cholest-4-en-3-one-7α-ol is formed, as has been discussed previously (p.125). However, on adding 3 ml. of SF to mitochondria, 10 μ moles of NAD and 7α-hydroxycholesterol only about 2% of the substrate is converted to cholest-4-en-3-one-7α-ol. (This has been measured in twelve experiments.)

By viewing the fluorescent plates obtained from incubations involving mitochondria and NAD, and mitochondria, NAD, and SF under the u.v. lamp, (Fig.21) a u.v. absorbing substance was seen to be formed in the incubation containing SF. This substance is slightly more polar in the benzene:ethyl acetate:acetone system than 7α-hydroxycholesterol having and R_F value of 0.31, whereas 7α-hydroxycholesterol has an R_F value of 0.35. On spraying the plate

Ultraviolet absorption spectrum of 'polar ultraviolet absorbing material'

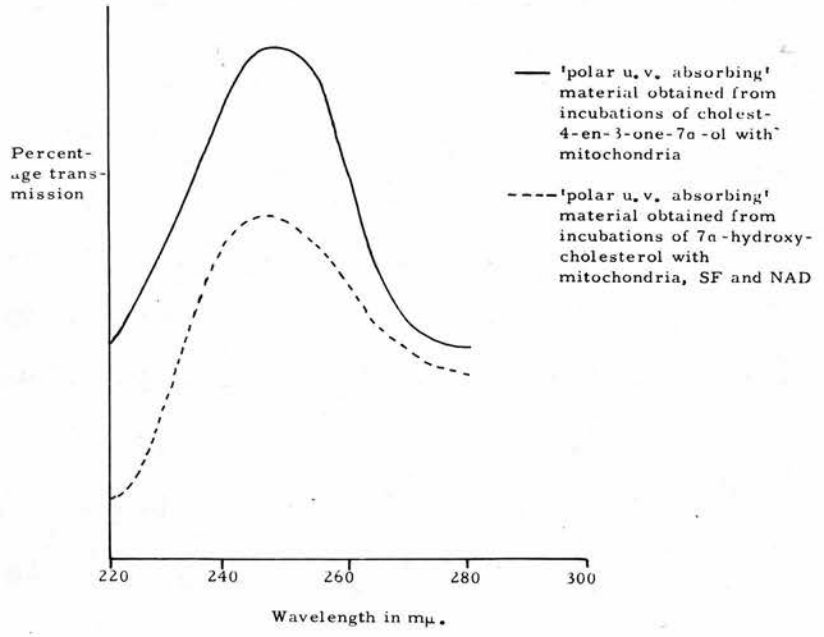
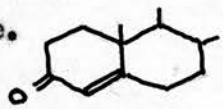


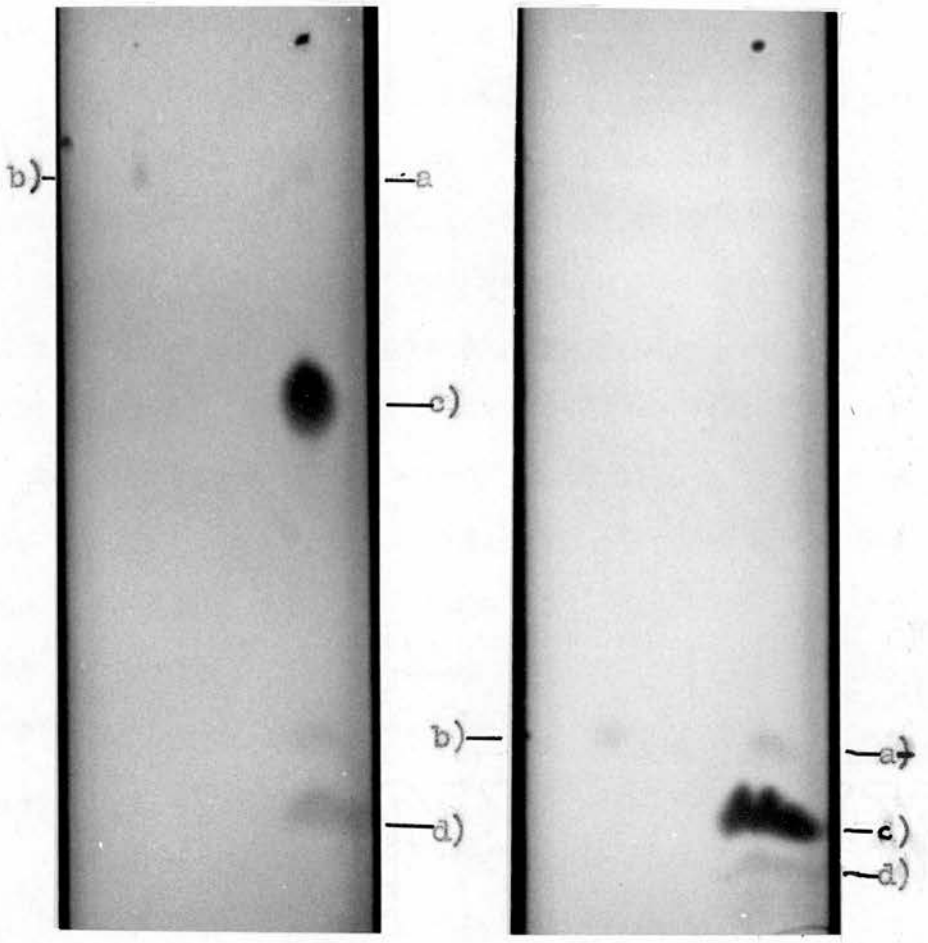
Fig. 23

with phosphotungstic acid, the substance gives a pink colour. When eluted from the plate and its u.v. absorption plotted, (Fig. 23), the substance was found to have a peak at 240 mμ., indicating that it is probably an α β -unsaturated ketone. On the basis of its mobility and properties it is suggested that this substance is a hydroxylated derivative of cholest-4-en-3-one-7α-ol, and that it is probably the 26-hydroxylated derivative (Danielsson, 1961 (e)).

It was not possible to separate this u.v. absorbing substance completely from 7α-hydroxy-cholesterol in the benzene:ethyl acetate:acetone system. It is therefore not possible to estimate it by radioactive measurements. If, however, it can be assumed that it has the same extinction co-efficient as cholest-4-en-3-one-7α-ol, which is probable because most Δ_4-3-ketones, ie.



molar
have/extinction co-efficients of about 16,000, it would be possible to estimate it by its absorption at 240 mμ. Such estimates showed that about 1% of the u.v. absorbing substance was formed.



A B

Fig. 24

Photographs of thin layer plates, obtained from incubations of mitochondria with 7α-hydroxycholesterol, with no NAD added. The plates were sprayed with phosphotungstic acid and plate A was run in benzene:ethyl acetate:acetone (10:5:3) while plate B was run in benzene:ethyl acetate:acetone:ethanol (10:5:3:2)

Substances present on plates:

a) incubation products (triol?)
b) standard cholest-5-en-3β,7α,26-triol
c) 7α-hydroxycholesterol
d) cholesterol

When the two incubations described above were sprayed it was discovered that a further metabolite was formed in the incubation with added SF. This substance gave a blue colour when the plate was sprayed with phosphotungstic acid, which would indicate that its structure is very similar to that of 7α-hydroxycholesterol. It is, however much more polar that 7α-hydroxycholesterol in the benzene:ethyl acetate:acetone system, and is also more polar than the u.v. absorbing substance also formed in this incubation. From a study of its mobility and properties it is suggested that the substance is cholest-4-en-3β,7α-26-triol, i.e., the 26-hydroxylated derivative of 7α-hydroxycholesterol (Danielsson, 1961 (e)). This polar product has the same mobility as the authentic cholest-5-en-3β-7α,26-triol (for synthesis, see Appendix, p. 307) in two thin layer systems. In benzene:ethyl acetate:acetone (10:5:3) both the product and the authentic substance have an R_F value of 0.15, and in benzene:ethyl acetate: acetone:ethanol (10:5:3:2) they have an R_F value of 0.78 (Fig. 24). Authentic cholest-4-en-3β, 7α,26-triol also gives a blue colour with phosphotungstic acid, as does the polar product. It

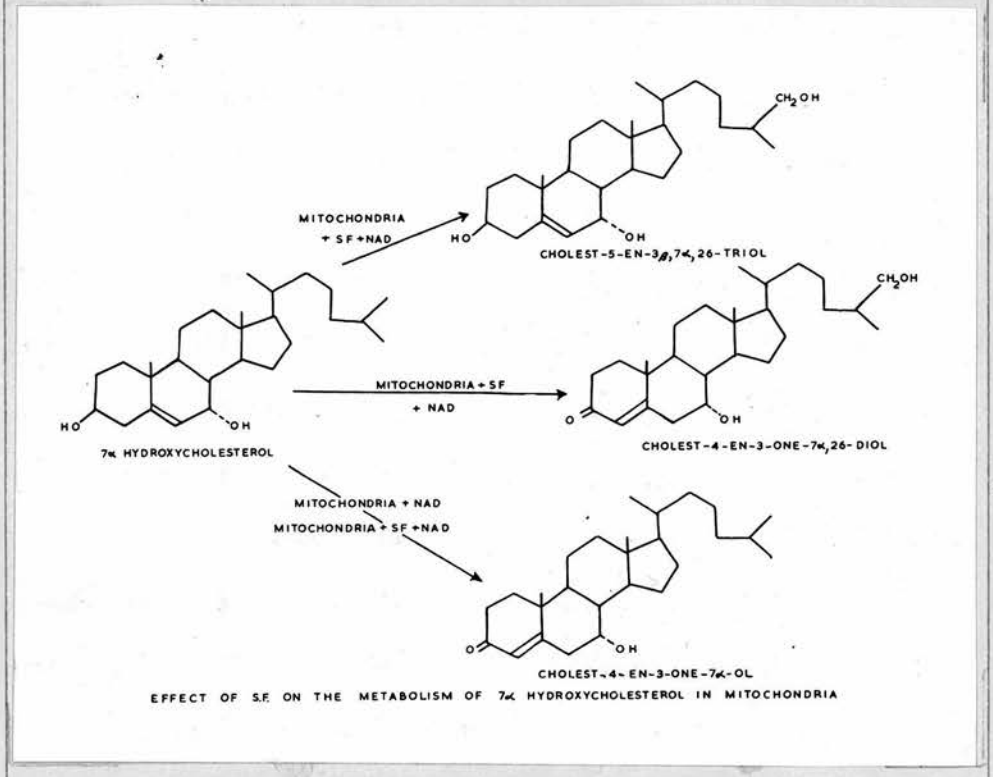
therefore seems very probable that the polar product is the 26-hydroxylated derivative of 7α-hydroxycholesterol.

When labelled 7α-hydroxycholesterol was used as substrate this metabolite was produced labelled and usually 3 - 4% was formed in incubations with mitochondria, NAD, and SF. This was measured in twelve experiments.

On some occasions a further metabolite can be seen in such incubations in minute quantities. This substance has a mobility which causes it, in the benzene:ethyl acetate:acetone system, to run between the cholest-4-en-3β,7α,26-triol and the cholest-4-en-3-one-7α,26-diol. It also gives a blue colour when sprayed with phosphotungstic acid, indicating that it has a structure similar to that of 7α-hydroxycholesterol.

In any further discussion of the two polar metabolites cholest-4-en-3-one-7α,26-diol and cholest-5-en-3β,7α,26-triol mentioned above they will be referred to as "polar u.v. absorbing substance" and "triol" respectively.

7-ketocholesterol was formed from 7α-hydroxycholesterol in incubations with mitochondria, NAD and SF, to the same extent as in incubations with



EFFECT OF S.F. ON THE METABOLISM OF 7α HYDROXYCHOLESTEROL IN MITOCHONDRIA

Figure 25

mitochondria and NAD, and as it is formed also in the boiled control incubation it again must be produced non-enzymically.

On running the concentrated ether cuts from the alumina columns from incubations with mitochondria, NAD and SF, the same substance as was formed in incubations with supernatant fraction and 7α-hydroxycholesterol was produced, i.e., an ester of 7α-hydroxycholesterol. This substance was found to be labelled when labelled 7α-hydroxycholesterol was used, and usually about 3% was formed. Very occasionally this ester is produced when 7α-hydroxycholesterol is incubated with mitochondria and NAD, but it always seems to be formed when SF is added to the mitochondria.

The diagram (Fig. 25) summarises the metabolites which are formed enzymically when 7α-hydroxycholesterol is incubated with mitochondria and mitochondria + SF, NAD having been added to both these fractions.

Thus, when SF is added to an incubation with mitochondria and NAD, the amount of cholest-4-en-3-one-7α-ol is decreased to less than half that, which would have been formed if no SF was present.

At least two (and probably three) further metabolites of 7α-hydroxycholesterol are produced by introducing SF, and as these are probably hydroxylated derivatives, it may be that SF plays some part in the complicated mechanism of hydroxylation. 7α-hydroxycholesterol ester may be produced on addition of SF because of the consequent addition of free fatty acids, or perhaps acyl coenzyme A esters.

The increase in disappearance of 7α-hydroxycholesterol from incubations on the addition of SF, as discussed in (d, i) cannot be accounted for by these differences in the metabolites produced. On a radioactive analysis, the percentage of 7α-hydroxycholesterol converted to metabolites in incubations with mitochondria and NAD and with mitochondria, NAD and SF, is almost the same. The amount of cholest-4-en-3-one-7α-ol formed in the first type of incubation is about 6%, whereas in the second type of incubation addition of the amounts of cholest-4-en-3-one-7α-ol, triol and u.v. absorbing material formed also equals about 6 - 7%. If ester is produced in the latter type of incubation it will only account for 3% of the added 7α-hydroxycholesterol.

Thus the 15% difference found between the disappearance of 7α-hydroxycholesterol when incubated with mitochondria and NAD and with mitochondria, NAD and SF, cannot be due to an increase in the formation of any metabolite but must be due to binding of the 7α-hydroxycholesterol or metabolites, promoted by SF, to the tissue.

(e) Attempt to discover the mechanism of the influence exerted by SF on the metabolism of 7α-hydroxycholesterol in mitochondria

In the previous Section the effect of adding SF to an incubation containing mitochondria, NAD and 7α-hydroxycholesterol has been discussed. It seems that SF somehow "directs" the metabolism of 7α-hydroxycholesterol so that hydroxylation rather than oxidation reactions are favoured.

(i) Incubation of 7α-hydroxycholesterol with mitochondria - no added NAD

Incubations were set up with mitochondria and 7α-hydroxycholesterol with no additions except buffer, magnesium sulphate and nicotinamide, i.e., no NAD was added at all. When the thin layer plates obtained from such incubations were examined under the u.v. lamp, no cholest-4-en-3-one-7α-ol was detected. 7-ketocholesterol was seen, but no polar u.v. substance was present. However, when

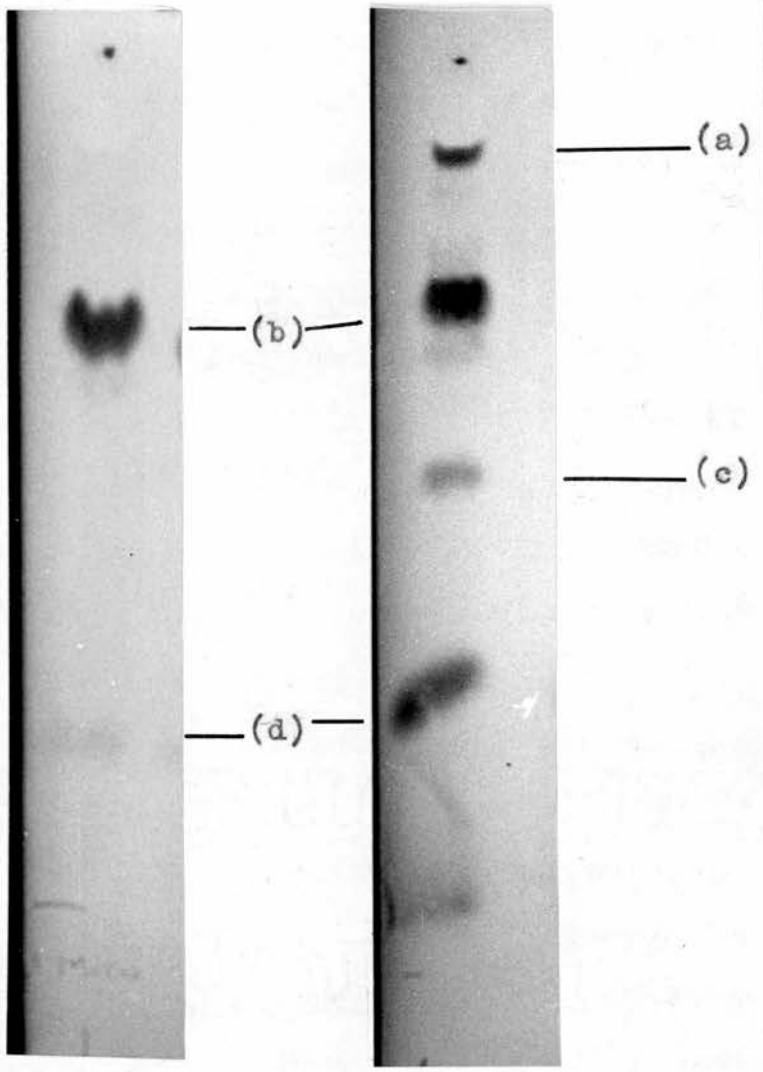


Fig. 26.

Photographs of thin layer plates, sprayed with phosphomolybdic acid.

1. Plate obtained from an incubation of 7α-hydroxycholesterol with mitochondria and NAD.

2. Plate obtained from an incubation of 7α-hydroxycholesterol with mitochondria, with no NAD added.

Substances appearing on plates:

a) Triol.

b) 7α-Hydroxycholesterol.

c) 7-Ketocholesterol.

d) Cholesterol.

the plates were sprayed with phosphotungstic acid, it was seen that cholest-5-en-3β,7α,26-triol had been formed (Fig. 26). This was labelled when labelled substrate was used, showing that it had been produced from the substrate.

These results show that if no NAD is added to incubations with mitochondria and 7α-hydroxycholesterol, hydroxylation of 7α-hydroxycholesterol occurs and no oxidation reactions take place. This situation, then, is similar to an extreme case of adding SF to mitochondria and NAD, when hydroxylation is favoured in place of oxidation. It would seem, therefore, that SF is exerting at least part of its influence by depleting in some way the added NAD so that the latter is no longer available for the oxidation of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol. Thus, hydroxylation becomes the favoured reaction. To test this hypothesis incubations were carried out in which mitochondria were incubated with 7α-hydroxycholesterol in the presence of increasing amounts of NAD

(ii) <u>Incubations of 7α-hydroxycholesterol with mitochondria - increasing NAD concentration</u>

Six incubations were set up each containing mitochondria and 7α-hydroxycholesterol (unlabelled)

and the NAD concentration was varied from zero to 50 μ moles NAD per 6 ml. of incubation mixture. An incubation containing mitochondria, 10 μ moles NAD and SF was also included. The thin layer plates obtained were viewed under the u.v. lamp and the cholest-4-en-3-one-7α-ol formed was marked eluted from the plates and estimated by its absorption at 242 mμ. The plates were then sprayed. Table 21 shows that the amount of cholest-4-en-3-one-7α-ol appearing per hr. is dependent on the NAD concentration. Judging the amount of triol formed by eye, it can be seen that as the NAD concentration increases the amount of triol formed decreases. Thus a considerable amount of triol was present at 2.5 μ moles NAD, and only a trace at 5 μ moles NAD.

This mitochondrial preparation seemed to be rather inactive at producing cholest-4-en-3-one-7α-ol. For example, when 10 μ moles NAD was added only 3 μg. cholest-4-en-3-one-7α-ol was formed. Usually in such a preparation this value is about 10 μg. However, this preparation seemed to be very active at producing the triol. The incubation which contained SF and 10 μ moles NAD produced triol, the polar u.v. absorbing substance and a trace of cholest-4-en-3-one-7α-ol.

TABLE 21

Mitochondria

Variation in NAD concentration

Substrate = 140 µg. 7α-hydroxycholesterol
(specific activity = 500 c/m/µg.)

Experiment (1)

NAD concentration (µ moles)	µg. cholest-4-en-3-one-7α-ol formed	Triol formed
0	0	+++
2.5	0	++
5	0.6	+
10	3	-
50	20	-

Experiment (2)

NAD concentration (µ moles)	Cholest-4-en-3-one-7α-ol formed (c/m)	Triol formed (c/m)
0	400	2700
5	2300	1400
10	3400	770
20	5675	600
40	7330	500

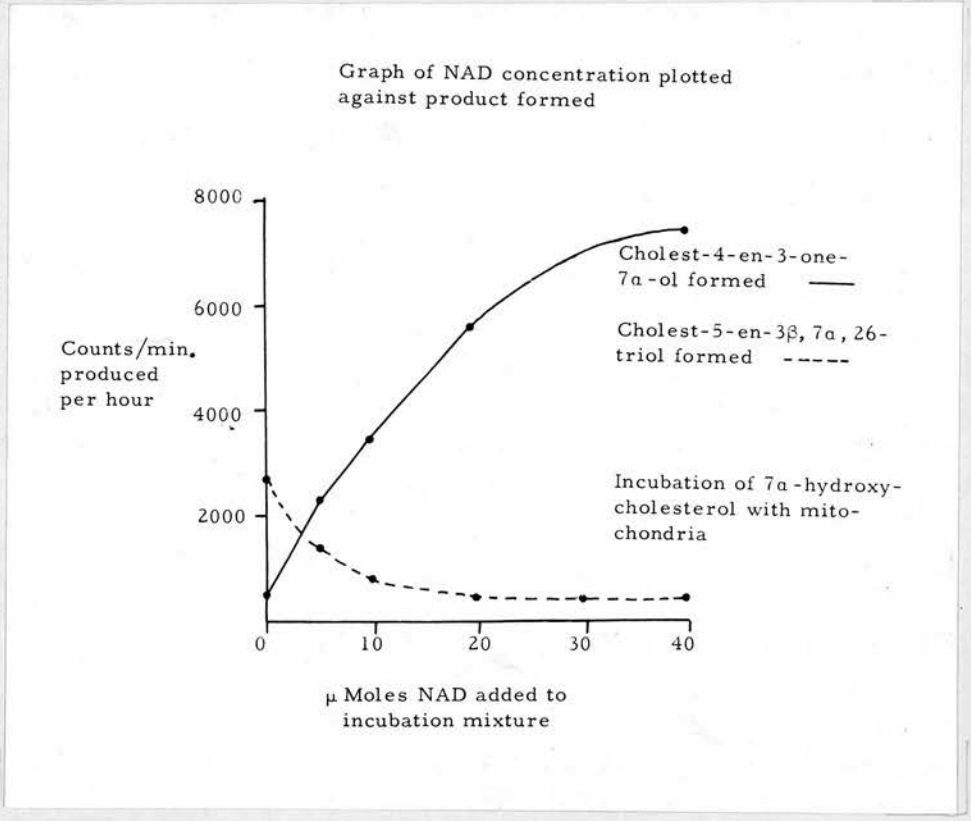

Graph of NAD concentration plotted against product formed
8000
6000
4000
2000
Counts/min. produced per hour
Cholest-4-en-3-one-7α-ol formed
Cholest-5-en-3β, 7α, 26-triol formed
Incubation of 7α-hydroxy-cholesterol with mito-chondria
0
10
20
30
40
μ Moles NAD added to incubation mixture

Figure 27

The experiment was repeated using labelled substrate. In this case the cholest-4-en-3-one-7α-ol formed and the triol formed were estimated by radioactive means. From Table 21 and the accompanying graph (Fig. 27) it can be seen that as the NAD concentration increases, the amount of triol formed decreases and the amount of cholest-4-en-3-one-7α-ol formed increases, confirming the results of the above experiment. The values in this experiment are more typical than those in the one described above.

These results, therefore, support the hypothesis that SF may act by depleting the added NAD available for the oxidation reaction. These results do not explain, however, how or why the polar u.v. absorbing substance is formed when SF is added to mitochondria and NAD.

The difference in apparent 3β-hydroxy dehydrogenase and isomerase activity in the two experiments just described can be explained if the levels of endogenous NAD in the mitochondria when prepared were different. If the NAD concentration in the mitochondria used in the first experiment were much lower than normal it follows that more NAD has to be added to these mitochondria

than to the "normal" mitochondria in the second experiment to obtain the same degree of oxidation of 7α-hydroxycholesterol.

(iii) Incubation of 7α-hydroxycholesterol with mitochondria and NAD. Succinate concentration varied

If the hypothesis that SF exerts part of its influence by "inactivating" NAD is correct, then it is possible that it does so by reducing the added NAD to NADH. This would agree with the apparent direction by SF towards hydroxylation reactions, as the NADH so formed could be converted by a transhydrogenase to NADPH, which seems to be essential for most hydroxylation reactions.

Recent work by Chance (1956), Slater and Hülsmann (1959) and Snoswell (1962) has aroused interest in the possibility that dicarboxylic acids such as succinic acid, may control the ratio of NAD to NADH in the living mitochondrion. In fact, they have shown that succinate reduces NAD to NADH more efficiently than do the conventional NAD-linked Krebs cycle acids, such as malate. This is a different reaction from the normal Krebs cycle oxidation of succinate to fumurate, which is FAD-linked.

It was therefore decided to try adding succinate to incubations with mitochondria, NAD and 7α-hydroxycholesterol in place of SF, to discover whether succinate had the same effect as SF.

In a preliminary experiment, incubations were set up comparing mitochondria + 10 μ moles NAD, mitochondria + 10 μ moles NAD + SF, mitochondria + 10 μ moles NAD + 36 μ moles succinate, and mitochondria with no additions. On examining the thin layer plates it was seen that:-

(a) mitochondria + NAD produced only cholest--4-en-3-one-7α-ol;

(b) mitochondria + NAD + SF produced less cholest-4-en-3-one-7α-ol, but also produced triol and polar u.v. absorbing substance;

(c) mitochondria + NAD + succinate also produced less cholest-4-en-3-one-7α-ol but also produced triol and u.v. absorbing substance;

(d) mitochondria alone produced only triol

Thus it would seem that succinate can indeed mimic the effect of SF.

In order to investigate this matter more closely an experiment was set up using labelled substrate and incubating it with mitochondria + 10 μ moles NAD and varying succinate concentra-

1 2

Fig. 28

Photograph of a thin layer plate, sprayed with phosphotungstic acid. Cholest-5-en-3β,7α, 26-triol isolated from incubations of mitochondria with 7α-hydroxycholerol, 10 μ moles NAD and succinate, re-run in benzene:ethyl acetate:acetone.

1. 7.2 μ moles succinate
2. 36 μ moles succinate

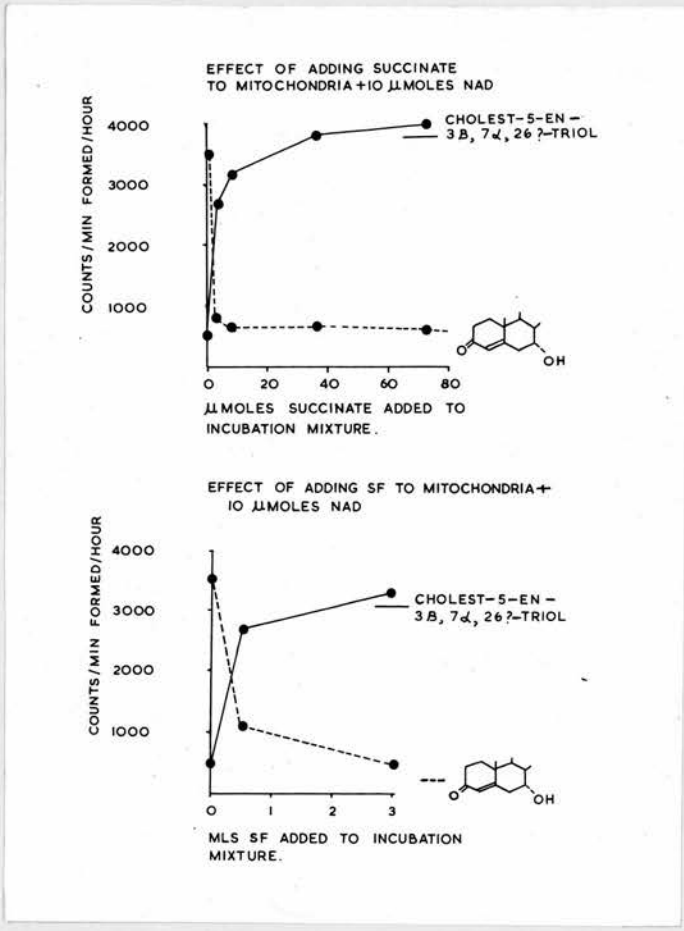

EFFECT OF ADDING SUCCINATE
TO MITOCHONDRIA + 10 μMOLES NAD
COUNTS/MIN FORMED/HOUR
4000
3000
2000
1000
CHOLEST-5-EN-
3β, 7α, 26?-TRIOL
OH
0
20
40
60
80
μMOLES SUCCINATE ADDED TO
INCUBATION MIXTURE.
EFFECT OF ADDING SF TO MITOCHONDRIA +
10 μMOLES NAD
COUNTS/MIN FORMED/HOUR
4000
3000
2000
1000
CHOLEST-5-EN-
3β, 7α, 26?-TRIOL
OH
0
1
2
3
MLS SF ADDED TO INCUBATION
MIXTURE.

Fig. 29

tion. Using mitochondria from the same preparation, incubations were set up with mitochondria + 10 μ moles NAD and with varying amounts of SF added. The cholest-4-en-3-one-7α-ol formed in each case was estimated by eluting it from the plates and reading its absorption at 242 mμ., after which it was also estimated by radioactive means. The triol formed was also eluted from the plates, an aliquot part taken for counting, and the rest re-run on thin layer plates in the benzene:ethyl acetate:acetone system. The plates were sprayed and then photographed in order to identify the triol, (Fig. 28)

From Table 22 and the accompanying graph (Fig. 29) it can be seen that adding a small amount of succinate (3.6 μ moles) gives a large degrease in the amount of cholest-4-en-3-one-7α-ol formed and an increase in the amount of triol produced. The amount of triol formed increases slightly after the initial sharp increase as succinate concentration increases and the amount of cholest-4-en-3-one-7α-ol formed decreases slightly.

The curves obtained by plotting increasing amounts of SF against, in the first instance, the amount of cholest-4-en-3-one-7α-ol formed, and

TABLE 22

Mitochondria

10 μ moles NAD added to each incubation

a) Variation in succinate concentration
b) Variation in amount of SF

a) μ moles succinate added	Cholest-4-en-3-one-7α-ol formed		Triol formed c/m
	μg.	c/m	
0	16.5	3500	400
3.6	1	735	2700
7.2	0.5	630	3140
36	0.5	700	3800
80	0	500	4000

b) ml. SF added	Cholest-4-en-3-one-7α-ol formed		Triol formed c/m
	μg.	c/m	
0	16.5	3500	400
0.5	2	1060	2700
3	0.5	548	3300

(140 μg. substrate (70,000 c/m) added to each incubation)

secondly the amount of triol formed, were of the same shape as those given by increasing succinate concentration.

The polar u.v. absorbing substance was formed in all incubations containing mitochondria, NAD and either SF or succinate, but was not formed in those containing mitochondria and NAD.

These results confirm those found in the preliminary experiment involving succinate and show that succinate has the same effect on triol formation, or direction of the reaction towards hydroxylation, as SF. This would indicate that SF is exerting its influence by depleting added NAD by reduction to NADH, but this does not mean that SF is necessarily succinate, although succinate does exist in the supernatant fraction of rat liver and is thermostable. Experiments performed later and described on pages 159-161 show that SF is probably not succinate.

(iv) Effect of various dicarboxylic acids on the metabolism of 7α-hydroxycholesterol in mitochondria with added NAD

If the hypothesis that succinate and SF direct the metabolism of 7α-hydroxycholesterol towards hydroxylation by reducing added NAD to NADH is

correct, other citric acid cycle intermediates should have the same effect. For example, fumarate should have a similar effect to succinate, but maleate (the cis-isomer of fumarate) should not.

(a) An experiment was set up to compare the effect of adding succinate, fumarate and maleate to mitochondria and NAD with labelled 7α-hydroxycholesterol as substrate. The cholest-4-en-3-one-7α-ol formed in each case was isolated by elution from thin layer plates and estimated by the absorption at 242 mμ. and by radioactive means. The amount of triol formed in each case was also determined by radioactive methods, an aliquot part being re-run on thin layer plates to identify the substance. The results are given in Table 23.

TABLE 23.

Incubation	cholest-4-en-3-one-7α-ol formed (c/m)	Triol formed (c/m)
i. Mitochondria + 10 μ moles NAD	2030	-
ii. As i + 72 μ moles succinate	350	2100
iii. As i + 72 μ moles fumarate	680	2000
iv. As i + 72 μ moles maleate	2200	-

(140 μg. substrate = 70,000 c/m, added)

It is thus seen that fumarate has the same effect as succinate when added to mitochondria + NAD, i.e., it lowers the amount of cholest-4-en-3-one-7α-ol formed and gives rise to triol formation. Fumarate, although not an NAD-linked substrate, must be stimulating the removal of NAD by the citric acid cycle. Maleate, on the other hand, has no "succinate-like" activity, which would be expected as it is not a citric acid cycle intermediate.

(b) A further experiment was carried out similar to that above, in order to compare the effect of adding SF, oxaloacetate and citrate to mitochondria, NAD, and 7α-hydroxycholesterol. An incubation with unwashed mitochondria and NAD was also included. The experiment was carried out exactly as above and the results are shown in Table 24.

TABLE 24

Incubation	cholest-4-en-3-one-7α-ol formed (c/m)	Triol formed (c/m)
i. Mitochondria + 10 μ moles NAD	3570	-
ii. As i + 3 ml. SF	2060	1400
iii. As i + 72 μ moles oxaloacetate	1630	1700
iv. As i + 72 μ moles citrate	1230	3300
v. Unwashed mitochondria + 10 μmoles NAD	5000	-

These results show that both oxaloacetate and citrate have the same effect on the metabolism of 7α-hydroxycholesterol in mitochondria as SF or succinate. In other words, adding citrate or oxaloacetate gives rise to less cholest-4-en-3-one-7α-ol than would be formed without these acids, and also triol and the "u.v. absorbing material" are formed. Citrate seems to be particularly active in this respect. Comparing unwashed and washed mitochondria with added NAD, shows that more cholest-4-en-3-one-7α-ol (7%) is formed in incubations of 7α-hydroxycholesterol with unwashed mitochondria, than is formed in washed mitochondria (5%). This fact is probably due to less endogenous NAD being present in washed mitochondria, some having been dialysed out during the washing procedure.

(c) A further experiment was carried out, similar to those described above, to compare the effect of adding SF, succinate, malonate, α-ketoglutarate and dihydroxyfumarate to incubations of 7α-hydroxycholesterol with mitochondria and NAD. The results are shown in Table 25.

TABLE 25

Incubation	Cholest-4-en-3-one-7α-ol formed (c/m)	Triol formed (c/m)
i. Mitochondria + 10 μ moles NAD	4800	-
ii. As i + 3 ml. SF	3220	600
iii. As i + 72 μ moles succinate	3500	800
iv. As i + 72 μ moles malonate	5500	-
v. As i + 72 μ moles α-ketoglutarate	3100	700
vi. As i + 72 μ moles dihydroxyfumarate	7470	-

These results are atypical, because, in this experiment, the mitochondria were not washed with sucrose. Thus, the amount of cholest-4-en-3-one-7α-ol formed in mitochondria + NAD is higher than usual, due to the fact that probably more endogenous NAD was present (see Table 24). SF, however, has its usual activity in lowering the amount of cholest-4-en-3-one-7α-ol formed, but very little triol, if any, is produced. The counting results for the triol formation could not be confirmed by running an aliquot on thin layer plates. Succinate and α-ketoglutarate are

seen to have the same effect as SF. Malonate and dihydroxyfumarate, however, exert no such effect and, in fact, give rise to more cholest-4-en-3-one-7α-ol than mitochondria and NAD.

The results of these three experiments strengthen the theory that SF has its effect partially by depleting the added NAD. The dicarboxylic acids and the tricarboxylic acid, citrate, having the same effect as SF are all citric acid cycle intermediates, (i.e. succinate, fumarate, α-ketoglutarate and oxaloacetate) and all stimulate the removal of NAD as NADH whereas the dicarboxylic acids having no effect are closely related acids, but are not citric acid cycle intermediates. The fact that malonate and dihydroxyfumarate actually stimulate the formation of cholest-4-en-3-one-7α-ol from 7α-hydroxycholesterol can be explained if these acids are inhibiting the normal functioning of the citric acid cycle, thus making more NAD "available" for reaction.

Incubations were carried out in order to compare the effect of NAD + SF and NADH on the various reactions involving 7α-hydroxycholesterol in mitochondria. It was found that NADH has

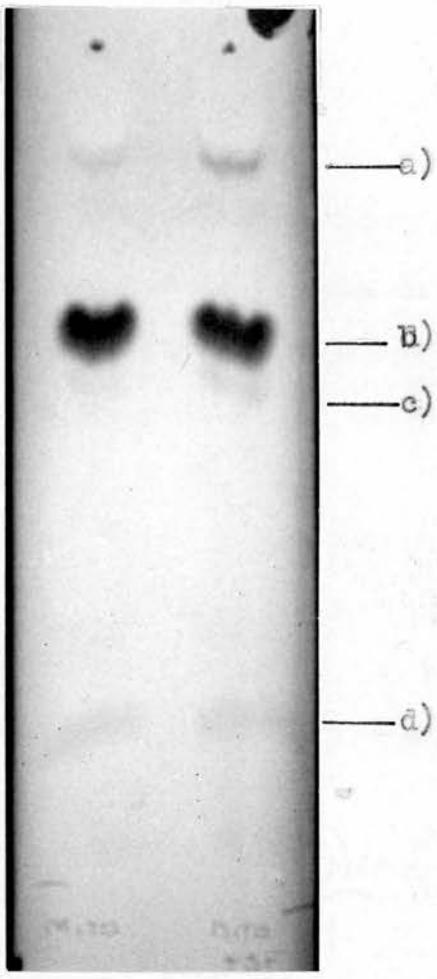


Fig. 30

Photograph of a thin layer plate obtained from incubations of 7α-hydroxycholesterol with mitochondria, sprayed with phosphotungstic acid.

1. Mitochondria and 7α-hydroxycholesterol with no NAD added
2. Mitochondria, 3 ml. SF, and 7α-hydroxycholesterol, with no NAD added.

Substances appearing on plate:

a) cholest-5-en-3β,7α,26-triol
b) 7α-hydroxycholesterol
c) 7β-hydroxycholesterol
d) cholesterol

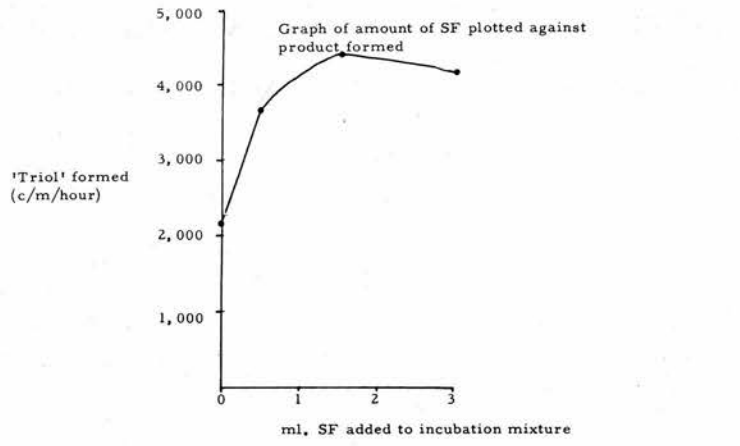
exactly the same effect as NAD + SF. In both cases, the amount of cholest-4-en-3-one-7α-ol formed was considerably less than that produced by mitochondria and NAD, and triol and the "uv. absorbing material" were also formed.

This evidence is further support for the theory that SF exerts an effect by reducing NAD to NADH.

(v) Incubations of 7α-hydroxycholesterol with mitochondria with no added NAD but varying the amount of SF added.

It had been noticed in previous experiments that the amount of triol formed when mitochondria alone were incubated with 7α-hydroxycholesterol was always less than the amount formed when SF was added to incubations with mitochondria and NAD (see Fig. 30). It therefore seemed possible that SF not only reduces the NAD to NADH but also in some way "stimulates" hydroxylation.

An experiment was therefore carried out in which mitochondria were incubated with 7α-hydroxycholesterol without any added NAD and with varying amounts of added SF. Labelled substrate was used. The triol formed in each case was eluted from the thin layer plates, an aliquot part taken for counting and the rest re-run on thin layer plates


Graph of amount of SF plotted against product formed
'Triol' formed (c/m/hour)
5,000
4,000
3,000
2,000
1,000
0
1
2
3
ml. SF added to incubation mixture

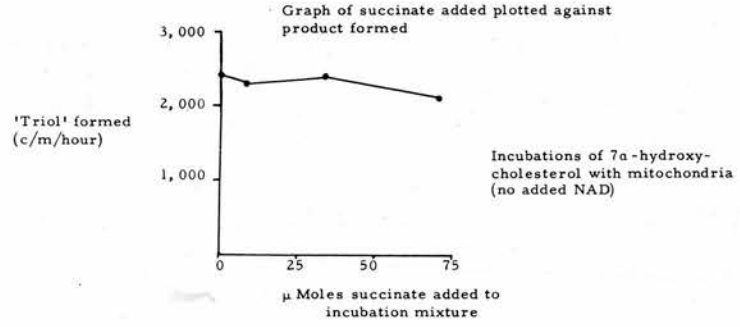

Graph of succinate added plotted against product formed
'Triol' formed (c/m/hour)
3,000
2,000
1,000
0
25
50
75
μ Moles succinate added to incubation mixture
Incubations of 7α-hydroxy-cholesterol with mitochondria (no added NAD)

Fig. 31

and sprayed with phosphotungstic acid to identify the triol.

From Table 26 and the accompanying graph (Fig. 31) it is seen that the amount of triol formed does in fact increase as the amount of SF added to the incubation is increased. (As no NAD was added, no cholest-4-en-3-one-7α-ol was formed).

TABLE 26

Incubation	Triol formed (c/m)
Mitochondria	2200
Mitochondria + 0.5 ml. SF	3700
Mitochondria + 1.5 ml. SF	4500
Mitochondria + 3 ml. SF	4200

(140 µg. substrate (70,000 c/m) added)

This increase in triol formation could be explained in two ways: either (a) the SF is "stimulating" hydroxylation or(b) the SF is reducing any endogenous NAD present.

If, as has been suggested, the reason that triol is formed is because less cholest-4-en-3-one-7α-ol is produced, the second explanation is

not a very likely one. In the last experiment no cholest-4-en-3-one-7α-ol was produced, which would indicate that the concentration of endogenous NAD is not sufficiently high for the reaction to occur.

(vi) Incubations of 7α-hydroxycholesterol with mitochondria with no added NAD and varying the succinate concentration

In section e (v) it was seen that with no added NAD, SF stimulated in some way the formation of triol. It was therefore of interest to discover whether succinate could replace SF in this respect as well as in incubations where NAD was included.

An experiment similar to that discussed in e (v) was set up, but in this case succinate was added in varying amounts (zero to 72 μ moles) instead of SF. Labelled 7α-hydroxycholesterol was used and the triol formed in each case estimated by radioactive means. The results are show in the Table below and Fig. 31.

TABLE 27

Incubation	Triol formed (c/m)
Mitochondria	2400
Mitochondria + 7.2 μ moles succinate	2300
Mitochondria + 36 μ moles succinate	2390
Mitochondria + 72 μ moles succinate	2120

These results show that when varying amounts of succinate are added to mitochondria and 7α-hydroxycholesterol no increase in the amounts of triol formed can be detected. Thus succinate, unlike SF, does not stimulate triol formation, but only acts by depleting the added NAD.

(vii) Substitution of NADP for NAD in incubations of 7α-hydroxycholesterol with mitochondria

Incubations were set up to compare the effect of NADP with that of NAD on the oxidation reaction occurring in mitochondria when 7α-hydroxycholesterol is converted to cholest-4-en-3-one-7α-ol as follows:-

(a) Mitochondria + 10 μ moles NAD

(b) Mitochondria + 5 μ moles NADP

(c) Mitochondria + 10 μ moles NADP

(d) Mitochondria + 10 μ moles NADP + SF

The thin layer plates obtained from these incubations were viewed under the u.v. lamp and the cholest-4-en-3-one-7α-ol which had been formed was marked, eluted, and estimated in each case by its absorption at 242 mμ. The remaining part of each plate was sprayed with phosphotungstic acid. The results of this experiment are summarised in the following Table.

TABLE 28

Incubation	µg. cholest-4-en-3-one-7α-ol formed per hr.	Triol formed	Polar u.v. absorbing substance formed
a) 10 µ moles NAD	10	-	-
b) 5 µ moles NADP	2	+	+
c) 10 µ moles NADP	5	-	-
d) 10 µ moles NADP + SF	0	+++	+

These results showed that NADP could partially replace NAD as co-factor in the oxidation reaction. Thus 10 µ moles NADP gave only half the amount of cholest-4-en-3-one-7α-ol given by 10 µ moles NAD. The formation of cholest-4-en-3-one-7α-ol depended on the amount of NADP present; thus 5 µ moles gave less product than 10 µ moles, and at 5 µ moles NADP a trace of triol and polar u.v. absorbing substance could be detected. In incubations with NADP and SF (as with NAD + SF) no cholest-4-en-3-one-7α-ol was formed, but triol and u.v. absorbing substance were produced.

It can therefore be concluded that NADP behaves in the same way as NAD in these reactions although it is not so efficient as acting as

co-factor for the oxidation of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol.

(viii) The stability of SF to pH changes

In order to obtain some information about the nature of SF it was decided to treat it with acid and alkali at 100°C. If SF is a dicarboxylic acid this treatment should make no difference to its effect on the metabolism of 7α-hydroxycholesterol. If, on the other hand, a phosphate ester or similar substance, such as ATP, is involved, this treatment would bring about hydrolysis, especially under alkaline conditions.

SF was treated in three ways:-

(i) Hydrochloric acid was added to the SF (3 ml.) which was then heated for 5 min. at 100°C. The SF was then neutralised with alkali (sodium hydroxide).

(ii) Sodium hydroxide was added to the SF, which was then heated as in (i). The solution turned bright yellow. It was then neutralised by adding hydrochloric acid.

(iii) The amount of sodium chloride formed by the above treatment was calculated, and this amount added in the appropriate volume of water to a further 3 ml. of SF.

Incubations were set up as follows:-

(a) Mitochondria + NAD + SF,

(b) Mitochondria + NAD,

(c) Mitochondria + NAD + acid-treated SF,

(d) Mitochondria + NAD + alkali-treated SF,

(e) Mitochondria + NAD + sodium chloride-treated SF

Using labelled substrate, the cholest-4-en-3-one-7α-ol and the triol produced were estimated as in previous experiments. The results are summarised in the Table below.

TABLE

Incubation	Cholest-4-en-3-one-7α-ol formed (c/m)	Triol formed (c/m)
a) NAD + SF	920	1600
b) NAD	3430	-
c) NAD + "acid-SF"	1610	1100
d) NAD + "alkali-SF"	3900	-
e) NAD + "NaCl-SF"	700	2300

From these results it can be seen:

(1) that adding sodium chloride has no effect on the activity of the SF, which reduces the amount of cholest-4-en-3-one-7α-ol that would have been

formed if SF had been absent (see b) and which gives rise to triol;

(2) that "acid treated" SF does not seem to be quite as active as untreated SF, although triol is produced and the amount of cholest-4-en-3-one-7α-ol is reduced;

(3) that "alkali-treated" SF on the other hand, seems to be completely lacking in the typical SF activity.

It would therefore seem that SF cannot be only succinate, or some citric acid cycle intermediate, but that its activity may depend on the presence of a hydrolysable substance such as ATP.

(ix) Incubations with an acetone powder of mitochondria

An acetone powder of mitochondria was prepared as described on p. 39 . Incubations with native mitochondria from the same rat were shown to be quite normal, i.e., when SF was added to mitochondria and NAD, a small amount of cholest-4-en-3-one-7α-ol was formed as well as triol and u.v. absorbing substances.

Incubations were carried out with acetone powder, dissolved in buffer, with 7α-hydroxycholesterol and NAD and with substrate, NAD, and SF. The cholest-4-en-3-one7α-ol produced in each case was estimated by radioactive means, and the remainder of the plates sprayed.

The results are shown in the Table below.

Incubation	cholest-4-en--3-one-7α-ol formed (c/m)	Triol formed	u.v absorbing substance
Acetone powder + NAD	4550	-	-
Acetone powder + NAD + SF	1700	-	-

It is thus seen that no hydroxylation apparently takes place, so that an acetone powder of mitochondria does not behave like native mitochondria in this respect. However, the addition of SF does decrease the amount of cholest-4-en-3-one-7α-ol formed, as it does with native mitochondria. The 3β-hydroxy dehydrogenase and isomerase activity of the mitochondria is not impaired by the acetone treatment.

(x) Effect of hydroperoxide of linoleic acid

It has been mentioned in the Introduction that hydroxylation reactions might involve a hydroperoxide, as an oxygen donor. It seems feasible, also, that the hydroperoxide of linoleic acid might play a role in the hydroxylation of cholesterol and related sterols. It was therefore decided to find out whether potassium linoleate or the hydroperoxide had any effect on the hydroxylation of 7α-hydroxycholesterol.

Formation of hydroperoxide

Two methods are available for the formation of this substance from methyl linoleate or the potassium salt of linoleic acid.

(i) The plant enzyme, lipoxidase, very readily converts potassium (or sodium) linoleate to the hydroperoxide. 1 ml. of solution of methyl linoleate was hydrolysed for 1 hr. under nitrogen with 1 ml. alcoholic potassium (or sodium) hydroxide. The ethanol was removed in vacuo and potassium hydrogen phosphate was added until the solution was pH 7.4. The potassium linoleate was used in this form in some incubations. The solution was made alkaline, pH 9.0, and was used as a substrate for the lipoxidase enzyme in buffer. As the

hydroperoxide has an absorption peak at 230 mμ. the formation of this substance could be followed by allowing the reaction to occur at room temperature in a cell of the recording spectrophotometer.

(ii) The hydroperoxide of linoleate can be produced by the method of Banks, Fazakerley, Keay and Smith (1959). 1 ml. methyl linoleate was dissolved in petrol ether, and shaken with 80% methanol:water; oxygen 95%: CO_2 5% was bubbled through the solution for three hours, the peroxide being extracted into the methanol layer as it was formed. The methanol layer was concentrated until it was a white oil. This oil, when tested with potassium iodide and acetic acid, liberated iodine, indicating the presence of the hydroperoxide.

a) Incubations were set up to discover whether potassium linoleate or the hydroperoxide (formed by the action of lipoxidase) could replace SF in incubations of 7α-hydroxycholesterol with mitochondria and 10 μ moles NAD. The cholest-4-en-3-one-7α-ol was isolated in each incubation by elution from the thin layer plates and estimated by the absorption at 242 mμ. and by radioactive means.

The triol formed was estimated by eluting and counting an aliquot part of the eluate. The triol was identified by re-running on thin layer plates. The results are given below.

TABLE 29

Incubation (all contain 10 μ moles NAD)	Cholest-4-en-3-one-7α-ol formed (c/m)	Triol formed (c/m)
a) Mitochondria	3580	-
b) Mitochondria + SF	1670	2440
c) Mitochondria + linoleate	4050	-
d) Mitochondria + hydroperoxide	3068	1700
e) Mitochondria + linoleate	3830	sprayed without elution -ve
f) Mitochondria + hydroperoxide	3800	-ve

In incubations d) and f) a polar u.v. absorbing material was seen to be formed, with a mobility similar to that of the triol. It was therefore more polar than the "polar u.v. absorbing material" suspected to be cholest-4-en-3-one-7α,26-diol. This material may have given rise to the 1700 c/m

found in incubation d). By chromatography no triol was seen to be formed.

As can be seen, the presence of neither linoleate nor the hydroperoxide affects the formation of cholest-4-en-3-one-7α-ol, whereas SF reduces the amount formed by half. Also linoleate and the hydroperoxide cannot give rise to triol. Thus, neither linoleate or its hydroperoxide can replace SF.

b) Incubations of 7α-hydroxycholesterol with an acetone powder of mitochondria, with added NAD, showed that addition of SF lowered the amount of cholest-4-en-3-one-7α-ol formed but did not give rise to triol. This would indicate that either the hydroxylating system is inactivated by the acetone treatment or that the acetone has extracted some material necessary for hydroxylation. An experiment was carried out to find out if the hydroperoxide of linoleate, made by the method of Banks, et al. (1959) could give the acetone powder the power to hydroxylate 7α-hydroxycholesterol.

Incubations were set up containing a buffer solution of an acetone powder of mitochondria 10 μ moles NAD and varying amounts of hydroperoxide

where 0.1 ml. of the aqueous solution was theoretically sufficient to provide enough oxygen to hydroxylate 7α-hydroxycholesterol. (This was estimated by titration with potassium iodide and thiosulphate).

The cholest-4-en-3-one-7α-ol was isolated and estimated by radioactive means. The plates were then sprayed with phosphotungstic acid to develop any triol formed.

TABLE 30

Incubation (all contained 10 μ moles NAD)	Cholest-4-en-3-one-7α-ol formed (c/m)	Triol formed
i) Acetone powder	4560	-ve
ii) As i + 0.1 ml. peroxide	1347	-ve
iii) As i + 0.2 ml. peroxide	1188	-ve
iv) As i + 0.5 ml. peroxide	890	-ve
v) As i + SF	1700	-ve

Thus, the peroxide cannot give an acetone powder of mitochondria the power to hydroxylate 7α-hydroxycholesterol. However, the amount of cholest-4-en-7α-ol formed decreases as the amount of peroxide present increases which is a situation similar to

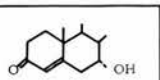
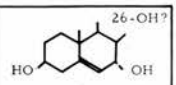
CELL FRACTION WITH ADDITIONS	 CHOLEST-4-EN — 3-ONE-7α -OL	 CHOLEST-5-EN — 3β, 7α, 26(?)-TRIOL
MITOCHONDRIA	-	3%
MITOCHONDRIA + 10 μmoles NAD	6%	-
+ 50 μmoles NAD	11%	-
MITOCHONDRIA + 10 μmoles NAD + SF	2%	4%
MITOCHONDRIA + SF (NO NAD ADDED)	-	6%
MITOCHONDRIA + 10 μmoles NAD + 10 μmoles CYANIDE	10%	-
MITOCHONDRIA + 10 μmoles NAD + 72 μmoles SUCCINATE	-	6%
ACETONE POWDER OF MITOCHONDRIA + 10 μmoles NAD	7%	-
ACETONE POWDER + 10 μmoles NAD + SF	2%	-

Fig. 32

that occurring when SF is added. As this lowering of the amount of product formed does not happen when native mitochondria are used, it is thought that the peroxide must be inhibiting the acetone powder enzyme. It may be that in native mitochondria, the hydroperoxide is rapidly metabolised.

The Table shown in Fig. 32 summarises the results obtained from incubations of mitochondria with 7α-hydroxycholesterol studied under various conditions.

(xi) Formation of polar u.v. absorbing substance

In many incubations carried out with mitochondria and 7α-hydroxycholesterol under various conditions it has been noted that the polar u.v. absorbing substance is only formed in those cases when triol is also produced, and co-factor is present. For example, the substance is formed in the following types of incubation:-

(a) Mitochondria + NAD (or NADP) + SF

(b) Mitochondria + succinate + NAD;

(c) Mitochondria + NADH;

(d) Mitochondria + 5 μ moles NAD (in this case the NAD concentration was low enough for triol to be produced; see p. 141).

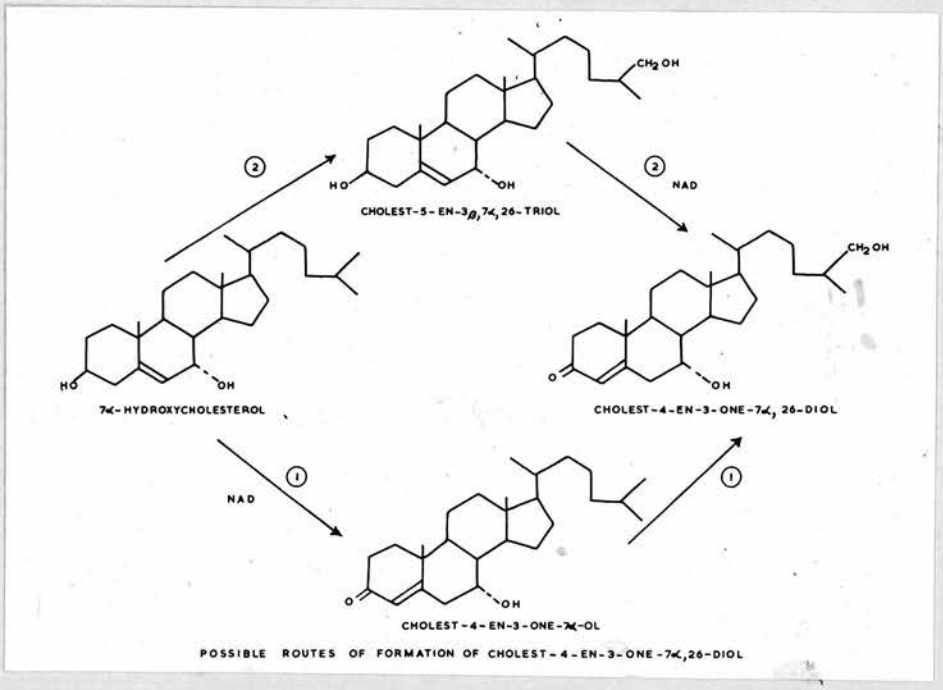

CH2 OH
HO
OH
CHOLEST-5-EN-3β,7α,26-TRIOL
2
2
NAD
7α-HYDROXYCHOLESTEROL
CHOLEST-4-EN-3-ONE-7α,26-DIOL
NAD
1
1
CHOLEST-4-EN-3-ONE-7α-OL
POSSIBLE ROUTES OF FORMATION OF CHOLEST-4-EN-3-ONE-7α,26-DIOL

Figure 33

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The polar u.v. absorbing substance is not, however, formed in the following types of incubation:-

(a) Mitochondria alone (triol is formed in this case)

(b) Mitochondria + SF (triol is formed in this case)

(c) Mitochondria + 10 μ moles NAD

This u.v. absorbing substance, if it is indeed cholest-4-en-3-one-7α,26-diol, could be formed by two routes:-

(i) by hydroxylation of cholest-4-en-3-one-7α-ol (this reaction does occur in mitochondria and will be discussed in the Section on the metabolism of cholest-4-en-3-one-7α-ol, p. 196)

(ii) by oxidation of the triol

The two routes are shown in Fig. 33.

If it were formed by hydroxylation of cholest-4-en-3-one-7α-ol it would be expected that more of it would be produced when more cholest-4-en-3-one-7α-ol was formed, such as when mitochondria were incubated with large amounts of NAD (20 - 40 μ moles). This, however, is not the case, as no polar u.v. substance is formed under these conditions.

It would seem that NAD or NADP is also required for its production, even if triol is formed, because mitochondria with no added NAD cannot give rise to the u.v. absorbing substance, although capable of producing triol. Thus it is probable that route (ii) is the correct one, and that the substance is formed by the oxidation of the triol for which presumably NAD or NADP is required. In the cases where SF or succinate are added to mitochondria + NAD and are thought to deplete the NAD it must be assumed that nevertheless sufficient is available for the oxidation of the triol to produce the polar u.v. absorbing substance.

Incubations with cholest-5-en-3β,7α,26-triol as substrate

Four incubations were set up with authentic cholest-5-en-3β,7α,26-tricl as substrate (about 20 µg.) in the following way

a) Mitochondria

b) Mitochondria + 10 µ moles NAD

c) Microsomes

d) Microsomes + 10 µ moles NAD

Comparing the fluorescent thin layer plates obtained from these incubations by viewing them

under the ultra-violet lamp showed that in incubation b) a u.v. absorbing substance with the same mobility in the benzene:ethyl acetate:acetone system (R_F = 0.31) as the u.v. absorbing material obtained from incubations of 7α-hydroxycholesterol with mitochondria, NAD and SF, was formed. No such substance was seen to be produced in incubation a). Therefore, mitochondria, on addition of NAD, can convert the triol to the "polar u.v. absorbing material" and presumably this is an oxidation reaction as it does not occur in the absence of NAD.

These results strengthen the theory described above, accounting for the formation of the "polar u.v. absorbing material" on incubation of 7α-hydroxycholesterol with mitochondria, NAD and SF, by an oxidation of the triol (see route (1), Fig. 33) rather than by hydroxylation of cholest-4-en-3-one-7α-ol. The identify of this "polar u.v. absorbing material" has been suggested to be cholest-4-en-3-one-7α,26-diol (p. 134) and the fact that it is found to be formed both by oxidation of the triol and by hydroxylation of cholest-4-en-3-one-7α-ol (see later, p. 196), greatly supports this suggestion.

A comparison of the plates obtained from incubations c) and d) showed that the u.v. absorbing material was produced from triol in both cases, although only a trace amount was seen in incubation c) which contained no NAD. Thus microsomes, particularly on addition of NAD, can also convert triol to the "polar u.v. absorbing material". This is not surprising as the microsomeshhave been found to convert 7α-hydroxycholesterol to the oxidised compound, cholest-4-en-3-one-7α-ol very efficiently in an analogous reaction and probably the same enzyme system is involved in the oxidation of the triol.

5. IDENTIFICATION OF THE METABOLITES PRODUCED ON INCUBATING 7α-HYDROXYCHOLESTEROL WITH MICROSOMES + SUPERNATANT AND MICROSOMES + SF

This subject is also discussed here instead of in its more logical place after p.104 because firstly the study was conducted after the work on mitochondria had been carried out, and secondly it is hoped that the discussion will be clearer if presented in this order.

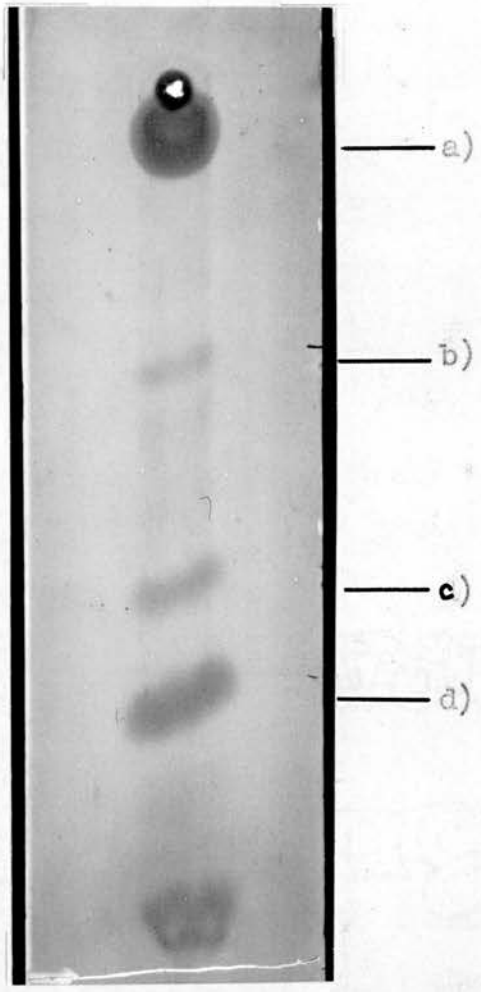


Fig. 34

Photography of fluorescent plate, illuminated by u.v. light. Plate obtained from an incubation of microsomes + supernatant with 7α-hydroxy-cholesterol and NAD

u.v. absorbing substances:-

a) nicotinamide
b) "polar u.v. absorbing material"
c) 7-ketocholesterol
d) cholest-4-en-3-one-7α-ol

A. Microsomes + supernatant with added NAD

Experiments were performed to compare the metabolites produced on incubation of 7α-hydroxycholesterol with microsomes + supernatant and with boiled microsomes + supernatant. 10 μ moles NAD was added to each incubation. The steroids were extracted and separated after incubation in the usual way, and the thin layer plates obtained were viewed under the u.v. lamp. As can be seen from the photograph (Fig. 34), three u.v. absorbing substances were formed in the active tissue incubation and only one in the boiled tissue control.

(a) The least polar substance formed in active tissue has the same R_F value (0.66) as cholest-4-en-3-one-7α-ol in the benzene:ethyl acetate: acetone system. It also has an absorption spectrum identical with that of this substance with a peak at 242 mμ. Cholest-4-en-3-one-7α-ol is presumably produced almost entirely by the microsomes with only 2% contributed by the supernatant.

(b) A substance with an R_F value of 0.56 in the benzene:ethyl acetate: acetone system was produced in both "boiled" and "active" incubations. This substance has properties identical with those of 7-ketocholesterol, and is presumably formed by autoxidation.

(c) The more polar u.v. absorbing substance discussed on p.104 was formed only in the active tissue incubation.

In spraying the plates obtained from incubations with active and boiled tissue no further metabolites could be seen. This was confirmed by carrying out a complete radioactive analysis of the ether:alcohol cut.

Examination of the thin layer plates obtained from the ether cuts from the columns revealed the formation of a substance less polar than 7α-hydroxycholesterol in active incubations. This substance was produced in incubations with supernatant alone and is thought to be an ester of 7α-hydroxycholesterol.

Thus it seems that microsomes + supernatant with NAD can give rise to the polar u.v. absorbing substance from 7α-hydroxycholesterol although neither microsomes and NAD, or supernatant and NAD can produce this substance. If the polar u.v. absorbing substance is the 26-hydroxy derivative of cholest-4-en-3-one-7α-ol, then active supernatant must contribute some factor or enzyme for hydroxylation.

B. Microsomes + SF, with added NAD

When microsomes were incubated with boiled, deproteinised supernatant (i.e., SF) and NAD, 7α-hydroxycholesterol was converted to cholest-4-en-3-one-7α-ol as expected. However, the polar u.v. absorbing substance was also produced. Thus, some thermostable factor in the SF somehow "activates" the microsomes giving them the power to hydroxylate.

This situation is similar to that occurring in mitochondria and it was wondered whether SF was acting by the same mechanism with microsomes as in mitochondria, i.e., SF appears to deplete added NAD.

If this were the case one would expect the following to occur:-

(a) Microsomes with no added NAD and therefore unable to produce cholest-4-en-3-one-7α-ol should be able to hydroxylate 7α-hydroxycholesterol to give triol.

(b) Microsomes incubated with SF and NAD should give rise to considerably less cholest-4-en-3-one-7α-ol than microsomes + NAD with no SF and triol should also be produced.

(c) Microsomes incubated with varying amounts of NAD should form, at low concentrations of NAD the two hydroxylated derivatives which occur in similar incubations with mitochondria.

(d) Adding succinate to incubations of microsomes with added NAD should decrease the amount of cholest-4-en-3-one-7α-ol produced from 7α-hydroxycholesterol and also give rise to the two hydroxylated derivatives, as in mitochondria.

Incubations were set up to discover whether (a) and (b) take place showing that neither of them occurred. Previous experiments (see pages 111 &117) also indicated that (c) and (d) do not occur. It thus appears that microsomes alone cannot hydroxylate 7α-hydroxycholesterol, and that SF, and also succinate, do not have the same effect as they do in mitochondria. Thus, the situation is not parallel to that occurring in mitochondria, and SF does not apparently deplete the added NAD.

C. Microsomes + supernatant with no added NAD

Microsomes + supernatant incubated with 7α-hydroxycholesterol without added NAD gave rise to no cholest-4-en-3-one-7α-ol, as expected. However, contrary to expectation, triol was seen to be formed. Thus, microsomes with active

supernatant and no NAD have the power to hydroxylate 7α-hydroxycholesterol. Incubations set up with active supernatant with no added NAD have shown that under these conditions no hydroxylation of 7α-hydroxycholesterol occurs (p.121).

D. Microsomes + SF with no added NAD

7α-hydroxycholesterol, when incubated with microsomes + SF, was not metabolised at all. No cholest-4-en-3-one-7α-ol was formed and also no triol.

Therefore in the formation of triol from 7α-hydroxycholesterol, a factor in the active supernatant fraction is required before microsomes are capable of hydroxylating 7α-hydroxycholesterol. This factor is probably enzymic, since on boiling and deproteinising the supernatant, this activity is lost.

These results indicate:-

Firstly, that if a thermostable factor contained in the supernatant fraction is added to incubations of 7α-hydroxycholesterol with microsomes and NAD, a polar substance is formed. It is suggested that this might be the 26-hydroxy

derivative of cholest-4-en-3-one-7α-ol. This substance is presumably formed by hydroxylation of cholest-4-en-3-one-7α-ol, as no triol is formed (see p. 176). This is quite a different situation to that occurring in mitochondria.
SF does not, apparently, direct the reaction towards hydroxylation by depleting the added NAD as it does in mitochondria;
secondly, that the addition of active supernatant to microsomes with no added NAD enables them to hydroxylate 7α-hydroxycholesterol to produce triol, indicating that two enzymes are probably involved, each inactive when confined to the separate cell fractions in which they are found.

SUMMARY OF SECTION IV

The metabolism of 7α-hydroxycholesterol was studied by incubating this substrate, both labelled with tritium and unlabelled, with the different cell fractions of rat liver. After incubation, a lipid extract was made and the constituents were crudely separated on small alumina columns. The metabolites were further separated, identified, and estimated by using thin layer chromatography on fluorescent thin layer plates.

It was found that:

(1) In the microsomes + supernatant fraction, the disappearance of 7α-hydroxycholesterol (used as a measure of its metabolism) was dependent on the concentration of NAD and also on substrate concentration.

(2) Anactive enzyme (or enzymes) was found to exist in microsomes which oxidised 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol. No other products were formed in this fraction.

This reaction was studied under various conditions:-

(i) It was found to be dependent on the NAD concentration, 30% of 7α-hydroxycholesterol being converted into cholest-4-en-3-one-7α-ol when 10 μ moles of NAD were added.

(ii) The reaction was dependent on substrate concentration

(iii) Increasing amounts of cyanide gave increasing amounts of product

(iv) The reaction had an optimal pH between 7.0 and 7.4

(v) Succinate had no effect on the reaction

(vi) An acetone powder of microsomes retained the enzymic activity.

(3) In the supernatant fraction, 7α-hydroxycholesterol was converted into a substance with properties and mobility similar to those of an ester of 7α-hydroxycholesterol. A trace of cholest-4-en-3-one-7α-ol was produced on adding NAD to the incubation mixture.

(4) The mitochondrial fraction of rat liver, in the presence of NAD could convert 7α-hydroxycholesterol into cholest-4-en-3-one-7α-ol.

a) This reaction was studied under various conditions:

(i) The formation of product was dependent on the substrate concentration.

(ii) Formation of cholest-4-en-3-one-7α-ol was dependent on the concentration of NAD added.

(iii) The addition of cyanide increased the amount of product formed.

b) The effect of SF on the metabolism of 7α-hydroxycholesterol in this fraction was studied.
(i) SF added to active mitochondria caused the substrate or products to be bound firmly to the tissue making complete recovery impossible. No such effect was noticed when SF was added to boiled mitochondria.

(ii) SF added to mitochondria, NAD, and 7α-hydroxycholesterol gave rise to the formation of a small amount of cholest-4-en-3-one-7α-ol and also to two other more polar metabolites, which, it is suggested, might be cholest-4-en-3-one-7α-26-diol and cholest-5-en-3β,7α,26-triol. A substance with mobility similar to that of an ester of 7α-hydroxycholesterol was also formed.

c) An attempt was made to discover the mechanims of the influence exerted by SF on the metabolism of 7α-hydroxycholesterol.

(i) Mitochondria without added NAD were found to convert 7α-hydroxycholesterol into the more polar "triol" compound.

(ii) Increasing the NAD concentration in incubations of mitochondria and substrate showed that as

the NAD concentration increased, the amount of "triol" formed decreased, and the amount of cholest-4-en-3-one-7α-ol formed increased.

(iii) The addition of increasing amounts of succinate to incubations of mitochondria, NAD, and substrate showed that succinate had the same effect as SF, in that as the succinate concentration increased the amount of "triol"formed increased, and the amount of cholest-4-en-3-one-7α-ol formed decreased.

(iv) Other dicarboxylic acids were incubated with mitochondria and NAD to find out if they could also mimic the effect of SF. Only those dicarboxylic acids which are citric acid cycle intermediates had the same effect as SF.

Conclusions were drawn from these experiments to explain the effect of SF. It is thought that SF may exert its effect partially by depleting in some way the added NAD, probably by converting it into NADH. NADH was found to have the same effect as NAD + SF.

(v) When increasing amounts of SF were added to mitochondria without added NAD, increasing amounts of "triol" were formed.

(vi) A similar experiment involving increasing the succinate concentration with mitochondria with no added NAD showed no such increase in "triol" formation. It was therefore concluded that SF may have two roles - depleting the NAD added, and "stimulating" hydroxylation reactions.

(vii) NADP was found to be able to replace NAD as co-factor for the oxidation reaction, although it was not as efficient. SF had the same effect when NADP was co-factor as when NAD was co-factor.

(viii) When the SF was treated with acid at 100°, and with alkali at 100°, and then brought back to neutrality and used in incubations it was found that acid-treated SF behaved in the same way as untreated SF, whereas alkali-treated SF lost its activity.

(ix) Incubations of 7α-hydroxycholesterol with an acetone powder of mitochondria showed that, on addition of NAD, the substrate was converted to

cholest-4-en-3-one-7α-ol. On addition of SF to such an incubation it was seen that no hydroxylation occurred, but less cholest-4-en-3-one-7α-ol was produced.

(x) Neither potassium linoleate nor its hydroperoxide could replace SF in incubations with native mitochondria. Also no hydroxylation of 7α-hydroxycholesterol was seen to occur on addition of linoleate hydroperoxide to an acetone powder of mitochondria.

(xi) It is thought that the polar u.v. absorbing substance must be formed from the "triol" in mitochondria by oxidation with NAD as co-factor.

(5) The metabolites produced in incubations of 7α-hydroxycholesterol with microsomes + supernatant, or microsomes + SF were discussed.

(A) Microsomes + supernatant with added NAD gave rise to the formation of cholest-4-en-3-one-7α-ol and also to the formation of the more polar u.v. absorbing substance, cholest-4-en-3-one-7α, 26-diol (?)

Approximate amounts of products formed during incubation of 7α-hydroxycholesterol with different cell fractions

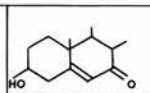
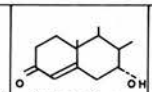
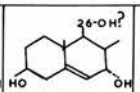
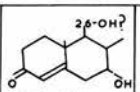
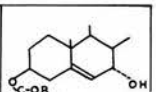
Cell Fraction	 7-Ketocholesterol	 Cholest-4-en-3-one-7α-ol	 Cholest-5-en-3β-7α-26?triol	 Cholest-4-en-3-one-7α-26?diol	 7α-Hydroxy-cholesterol ester
Mitochondria (with added NAD)	2.5%	6%	-	-	1%
Mitochondria + SF (with added NAD)	2.5%	2%	4%	1%	1%
Microsomes (with added NAD)	3%	25%	-	-	-
Supernatant	2%	2%	-	-	3%

Figure 35

(B) When SF was exchanged for supernatant in (A) the same result was obtained. Thus, a thermostable co-factor is present in supernatant which gives microsomes the power to hydroxylate cholest-4-en-3-one-7α-ol.

(C) Microsomes + supernatant with no added NAD gave rise to the formation of "triol".

(D) When SF was exchanged for supernatant in (C) it could not replace it, and no triol was formed. Thus, a thermolabile co-factor or enzyme is present in supernatant, giving microsomes the power to hydroxylate 7α-hydroxycholesterol.

SECTION V

METABOLISM OF CHOLEST-4-EN-3-ONE-7α-OL IN RAT LIVER CELL FRACTIONS

METABOLISM OF CHOLEST-4-EN-3-ONE-7α-OL

There is now much evidence to suggest that cholest-4-en-3-one-7α-ol is an intermediate in the breakdown of cholesterol to bile acids. Danielsson (1961, b) has shown that when the tritium-labelled compound is administered to rats with bile fistulas it is converted into bile acids, for the most part into chenodeoxycholic acid, and a little into cholic acid. The evidence presented here indicates that 7α-hydroxycholesterol is converted quite efficiently into cholest-4-en-3-one-7α-ol in the microsomal fraction of rat liver. If 7α-hydroxycholesterol is on the pathway from cholesterol to bile acids this evidence would support Danielsson's observations.

In order to investigate this point it was decided to incubate cholest-4-en-3-one-7α-ol unlabelled, and labelled with tritium, with the different fractions of rat liver, the object being to discover whether the substance was converted into 3α,7α-dihydroxycoprostane which has been shown by Bergström and Lindstedt (1956) to be an intermediate in the formation of bile acids.

Chemical synthesis of cholest-4-en-3-one-7α-ol

The chemical synthesis of cholest-4-en-3-one-7α-ol is described in the appendix (p. 304). The labelling of the pure compound with tritium and the subsequent purification of the radioactive compound are also outlined there.

Experimental conditions and methods

(a) The incubation mixture used in the following experiments was the same as that used in the incubations involving 7α-hydroxycholesterol as substrate, except that no NAD or other co-factors were added unless otherwise stated.

The incubation mixture was thus as follows:

200 μg. cholest-4-en-3-one-7α-ol (0.5 μ mole) in 0.1 ml. methanol

1 ml. 0.02 M phosphate buffer, pH 7.4

0.5 ml. 0.2 M nicotinamide (100 μ moles)

0.5 ml. 0.2 M magnesium sulphate (10 μ moles)

3.0 ml. tissue, equivalent to 1 g. wet weight of liver

(b) The extraction procedure used was that described on p. 50 and the dry residues were put on to 2 g. alumina columns in ether.

(c) Alumina columns

Two experiments were carried out, firstly to find out in which cut cholest-4-en-3-one-7α-ol was eluted from the 2 g. alumina column, and secondly to check the recovery of known amounts of cholest-4-en-3-one-7α-ol from these columns.

(i) For the estimation of cholest-4-en-3-one-7α-ol advantage was taken of its u.v. absorbing properties. Ethanolic solutions of the substance can be read in the u.v. spectrophotometer and from the optical density readings at 242 mμ. and the molar extinction co-efficient (15,500), the number of μ moles and thus of μg. of cholest-4-en-3-one-7α-ol in the solution can be calculated.

200 μg. cholest-4-en-3-one-7α-ol was put on a 2 g. alumina column in 5 ml. of ether. The column was eluted with a further 10 ml. of ether, then with 30 ml. of ether:alcohol (9:1) in two separate 15 ml. cuts and lastly with alcohol. The cuts were taken to dryness, and the residues run on thin layer fluorescent plates. The cholest-4-en-3-one-7α-ol was found to be eluted quantitatively in the ether:alcohol cut, mainly in the first 15 ml.

(ii) The recovery of cholest-4-en-3-one-7α-ol from the alumina columns was tested as follows:-

Amounts of a standard solution varying from 17 μg. to 176 μg. were taken to dryness and put on to 2 g. alumina columns in ether. The columns were eluted with 15 ml. of ether, and 30 ml. of ether:alcohol (9:1). The ether:alcohol cuts were taken to dryness and the residues dissolved in ethanol. The ethanolic solutions (3 ml.) were read at 242 mμ. and the number of μg. of the substance present calculated (see Table 31).

(d) Thin layer chromatography

Owing to the presence of nicotinamide in the ether:alcohol cuts (see p. 52) the cholest-4-en-3-one-7α-ol recovered from an incubation cannot be estimated directly in the ether:alcohol cut. The cuts off the alumina columns were therefore concentrated and run on fluorescent plates. Because of its u.v. absorbing properties, cholest-4-en-3-one-7α-ol is easily located on the plates and can therefore be eluted as described on p. 60 It can then be estimated either by its absorption at 242 mμ or by radioactive means.

TABLE 31

Recovery of Cholest-4-en-3-one-7α-ol From 2 g Alumina Columns

Cholest-4-en-3-one-7α-ol added to columns		Cholest-4-en-3-one-7α-ol recovered from columns		Percentage Recovery
Optical Density at 242 mμ.	μg.	Optical Density at 242 mμ.	μg.	
0.24	17	0.253	17	100
0.6	44	0.63	45	102
1.2	88	1.16	86	98
1.78	132	1.76	130	99
2.4	176	2.4	176	100

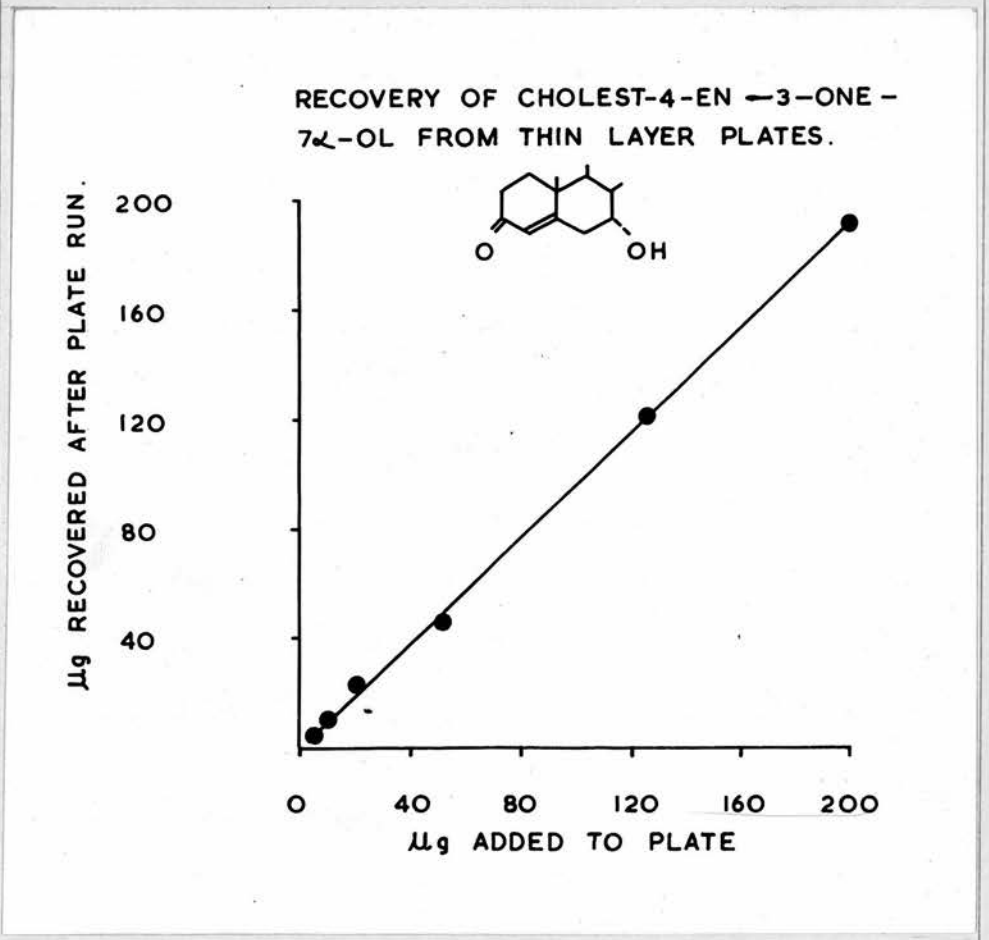

RECOVERY OF CHOLEST-4-EN —3—ONE —
7α—OL FROM THIN LAYER PLATES.
O
OH
µg RECOVERED AFTER PLATE RUN.
200
160
120
80
40
0
40
80
120
160
200
µg ADDED TO PLATE

Fig. 36

Recovery of cholest-4-en-3-one-7α-ol from thin layer plates

An experiment was carried out in which known amounts of labelled cholest-4-en-3-one-7α-ol from 5 μ g. to 190 μg. were spotted on to thin layer plates which were then run in the benzene: ethyl acetate: acetone system. The cholest-4-en-3-one-7α-ol was marked and eluted with 10 ml. of ether: alcohol (1:1). The extracts were taken to dryness and dissolved in ethanol. The ethanolic solutions were read at 242 mμ., taken to dryness and counted. The amounts of cholest-4-en-3-one-7α-ol recovered in this way were plotted against the amounts that were put on to the plates, as seen in Table 32 and Fig. 36.

Using the above methods, cholest-4-en-3-one-7α-ol, labelled and unlabelled, was incubated with the different cell fractions of rat liver, and its disappearance measured. Metabolites were also identified.

TABLE 32

Recovery of Cholest-4-en-3-one-7α-ol

From Thin Layer Plates

Cholest-4-en-3-one-7α-ol µg. added	Cholest-4-en-3-one-7α-ol µg. recovery	Percentage Recovery
5	6	120
10	9.2	92
20	21	105
50	45	90
127	120	95
190	190	100

A. MICROSOMAL FRACTION.

(i) Labelled cholest-4-en-3-one-7α-ol was incubated with "boiled" microsomes and "active" microsomes, the microsomes being prepared as described on p. 36. . The cholest-4-en-3-one-7α-ol was recovered from the thin layer plates and estimated by radioactive means. It was found that no cholest-4-en-3-one-7α-ol whatever had disappeared from either the incubation with active tissue or the boiled tissue control.

(ii) Identification of metabolites

Thin layer plates run on the ether:alcohol cuts obtained from incubations of cholest-4-en-3-one-7α-ol with boiled and active microsomes were viewed under the u.v. lamp and any u.v. absorbing spots were marked. The plates were then sprayed firstly with phosphotungstic acid and then with phosphomolybdic acid.

No further spots could be seen on the plate obtained from the incubation with active tissue which did not occur on the plate obtained from the boiled control incubation. 7α-hydroxycholesterol and 7β-hydroxycholesterol were seen to be produced on both plates. When a complete radio

active analysis was carried out on all the cuts off the alumina columns by segmenting the thin layer plates run on these cuts, it was found that all the radioactivity added was recovered as cholest-4-en-3-one-7α-ol from both "boiled" and "active" incubations. Thus no metabolism of cholest-4-en-3-one-7α-ol had occurred in the microsomal fraction. No radioactivity was associated with the 7α- and 7β-hydroxycholesterol which had been seen to be produced in both types of incubation, which indicates that these substances were produced by the autoxidation of the cholesterol occurring naturally in the microsomes and not from the added cholest-4-en-3-one-7α-ol. This was confirmed by carrying out incubations of active microsomes with no substrate present and showing that 7α-hydroxycholesterol and 7β-hydroxycholesterol were formed.

These experiments indicate that cholest-4-en-3-one-7α-ol is not metabolised further in the microsomal fraction of rat liver.

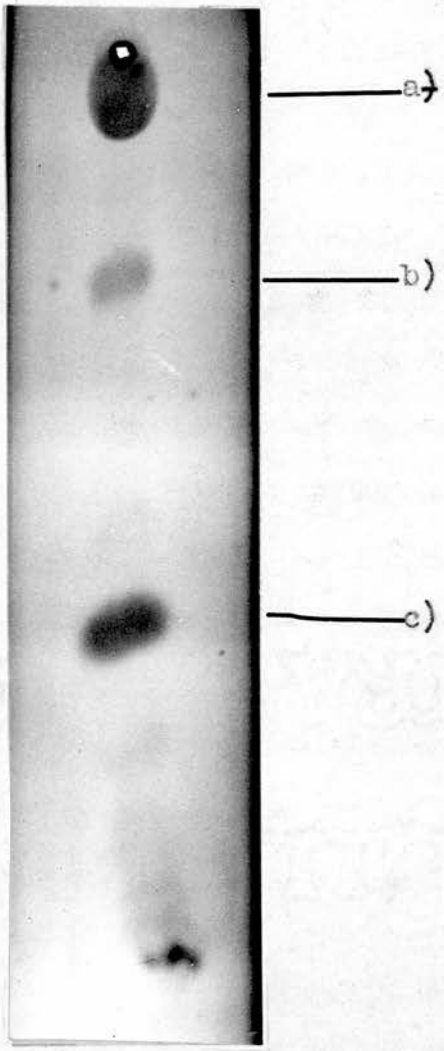


Fig. 37

Photograph of a fluorescent plate, illuminated by u.v. light. Plate obtained from an incubation of mitochondria with cholest-4-en-3-one-7α-ol

u.v. absorbing substances:-

a) nicotinamide
b) "polar u.v. absorbing material" (cholest-4-en-3-one-7α,26-diol?)
c) cholest-4-en-3-one-7α-ol

B. MITOCHONDRIAL FRACTIONS

(i) Cholest-4-en-3-one-7α-ol was incubated with "boiled" and "active" mitochondria, which were prepared as described on p.36. The substrate was recovered by elution from the thin layer plates and estimated. Using unlabelled substrate and comparing the recovery of substrate from incubations with boiled and active tissue it was found that in the active tissue incubation 10% of the cholest-4-en-3-one-7α-ol had disappeared (i.e. metabolised). When labelled substrate was used the disappearance in the active tissue incubation was found to be 6%, measured in three separate experiments.

(ii) Identification of metabolites

Thin layer plates run on the ether:alcohol cuts obtained from boiled and active tissue incubations were viewed under u.v. light and it was seen that in the active tissue incubation, but not in the boiled control, a u.v. absorbing substance had been formed, (Fig. 37). This substance had the same R_F value (0.31) in the benzene:ethyl acetate:acetone system as the "polar u.v. absorbing material" produced in incubations of 7α-hydroxy-

cholesterol with mitochondria + SF + NAD, mitochondria + succinate + NAD, and microsomes + SF + NAD (see p. 135). The substance, when eluted from the plate and its absorption spectrum plotted showed a peak at 242 mμ., indicating that it is probably an α,β -unsaturated ketone (Fig. 23). The substance behaves like cholest-4-en-3-one-7α-ol when sprayed with phosphotungstic acid, giving a pink colour. When labelled cholest-4-en-3-one-7α-ol was used as substrate, this u.v. absorbing material was found to be labelled, showed that it was formed from the substrate, about 4% being formed.

From the mobility of this u.v. absorbing material, and from the fact that it is formed from cholest-4-en-3-one-7α-ol it is suggested that the substance is the 26-hydroxylated derivative of the substrate, i.e., cholest-4-en-3-one-7α,26-diol. This is the suggested identity of the substance produced from 7α-hydroxycholesterol, (p. 135).

A complete radioactive analysis of the extracts from boiled control and active tissue incubations showed that no other radioactive products were formed in the active incubations. This was

supported by the fact that on spraying the plates no spots which were not present in the extracts from the boiled incubations appeared in the extracts from active incubations. Again 7α- and 7β-hydroxycholesterols were formed, but these were found not to be associated with any radioactivity. Very small amounts of 7α-&7β-hydroxycholesterols were formed and these separated completely from the polar u.v. absorbing material (compare p. 134 dealing with incubations of 7α-hydroxycholesterol).

(iii) The formation of the polar u.v. absorbing material studied under various conditions

(a) Effect of co-factors on the reaction

In many examples of hydroxylation reactions it has been found that NADPH is essential as a co-factor. Incubations were therefore set up to compare the effect of adding NADH and NADPH to mitochondria with cholest-4-en-3-one-7α-ol as substrate. The fluorescent plates obtained from such incubations were viewed under the u.v. lamp and it could be seen that the same amount of u.v. absorbing product was formed in incubations with mitochondria, mitochondria + NADH and mitochondria with NADPH. Thus it would seem that neither NADH or NADPH stimulate the reaction to give more of the u.v. absorb-product.

(b) Effect of adding boiled supernatant (or SF) to the incubations.

If SF does have some effect on hydroxylation, as has been inferred in the previous Section, then it should stimulate this reaction in which the more polar u.v. absorbing substance is formed from cholest-4-en-3-one-7α-ol.

Two separate experiments were carried out in which incubations were set up as follows, using labelled substrate:-

(a) Boiled mitochondria + 3 ml. SF

(b) Active mitochondria

(c) Active mitochondria + 3 ml. SF

(d) Active mitochondria + 6 ml. SF

The ether:alcohol cut from each incubation was run on a fluorescent thin layer plate, which was then segmented, and the segments eluted and counted. The results of the two experiments are shown in Table 33 . The ether and alcohol cuts from the columns were also analysed by radioactivity measurements and the total counts recovered from each incubation was found. These are shown in the Table as percentages of the number of counts added to the incubations.

TABLE 33

Incubation	Amount of polar u.v. substance formed in c/m		Percentage counts recovered	
	Expt.1	Expt.2	1	2
a)Boiled mitochondria + 3 ml. SF	200	104	97	96
b)Mitochondria	2400	3672	94	95
c)Mitochondria + 3 ml. SF	2100	4440	77	70
d)Mitochondria + 6 ml. SF	2000	4320	77	74

The results indicate that in experiment 1 the addition of SF makes no difference to the amount of polar u.v. absorbing substance formed, whereas in experiment 2 the addition of SF seems to produce slightly more of the substance. The results although inconclusive, have some interest. When the total number of counts recovered from the incubations is calculated, it is found that about 95% of the added counts can be recovered from the incubations with boiled mitochondria + SF, or from active mitochondria, whereas only about 75% of

the added counts can be recovered from incubations involving active mitochondria + SF. This is the same sort of situation as arose with incubations of 7α-hydroxycholesterol with mitochondria and SF, described on p.131 . It may be, then, that SF does bind, in some way, the substrate or products very firmly to the tissue.

(c) Effect of altering the gas phase in the incubation flasks

In many examples of hydroxylation reactions oxygen has been shown to be a necessary addition. It was therefore decided to incubate mitochondria and cholest-4-en-3-one-7α-ol in air, in a mixture of 95% oxygen + 5% carbon dioxide, and in nitrogen. Just before the incubation, the flasks were placed in the bath at 37°, after which they were flushed out for two or three minutes with the gases concerned, and then stoppered tightly. The flask to be incubated with air as the gaseous phase was stoppered as usual.

The polar u.v. absorbing substance produced was viewed on the fluorescent plates under the u.v. lamp and it was found that perhaps slightly more of the product was formed in the oxygen: carbon

dioxide medium, whereas in the nitrogen medium about the same amount was formed as in the incubation carried out in air.

Thus, oxygen may stimulate the reaction, and the fact that the nitrogen has no effect probably means that either the system is not anaerobic or that a bound-form of oxygen is present. Obviously more rigid anaerobic conditions must be applied.

(d) Disruption of the mitochondria

It was thought to be of interest to discover whether the reaction occurring in mitochondria and giving rise to the polar u.v. absorbing substance from cholest-4-en-3-one-7α-ol was sensitive to disruption of the mitochondria by sonication.

(i) An experiment was set up to compare the amount of polar u.v. absorbing material produced from cholest-4-en-3-one-7α-ol in native mitochondria with that produced in mitochondria (from the same preparation)which had been subjected to sonic disintegration. It was found that this treatment made no difference to the activity of the mitochondria, as can be seen from the photographs (Fig. 38), and also that the addition of NADPH to either native mitochondria or disrupted mitochondria gave no increase in the amount of u.v. absorb- product formed.

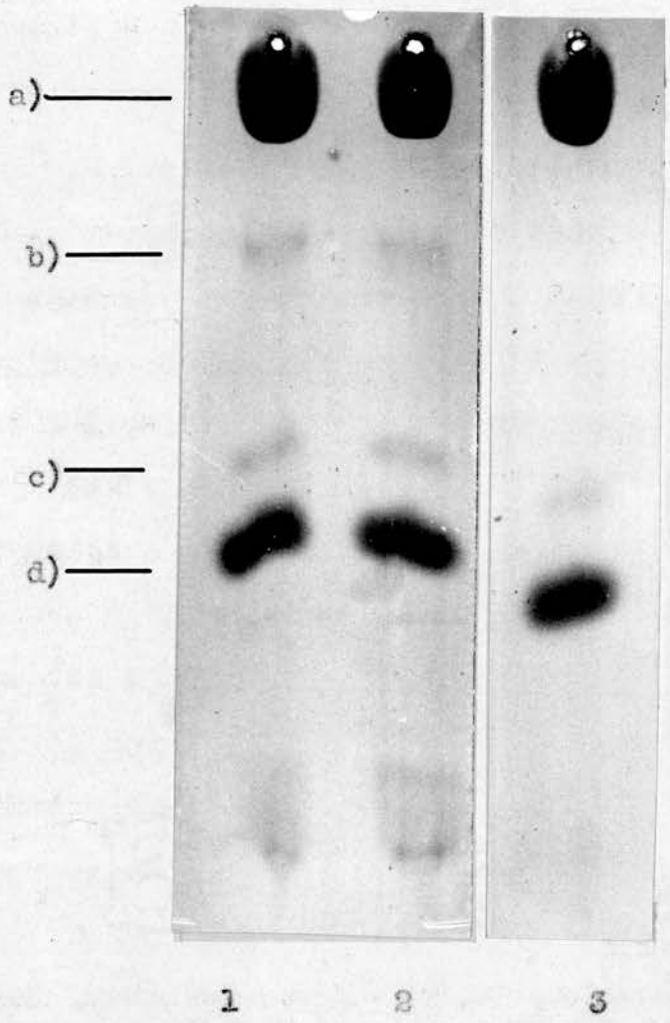


FIG. 38

Photographs of fluorescent plates, illuminated by u.v. light. Plates obtained from incubations of mitochondria with cholest-4-en-3-one-7α-ol

1. Native mitochondria
2. Mitochondrial debris, from sonication
3. Supernatant, from mitochondrial debris.

u.v. absorbing substances:-

a) nicotinamide
b) "polar u.v. absorbing material"
c) 7-ketocholesterol
d) cholest-4-en-3-one-7α-ol

(The fact that the mitochondria had indeed been disrupted after sonication was confirmed by examination under the interference microscope).

(ii) A sucrose solution of mitochondria which had been subjected to sonication was centrifuged at 10,000 x g for 20 min. in the M.S.E. refrigerated centrifuge. The debris was sedimented in this way and separated from the supernatant fraction which contained the soluble material from the broken mitochondria. The debris was suspended in sucrose solution and incubations were set up to compare the activity of the mitochondrial debris with that of the "supernatant". Native mitochondria from the same preparation were also incubated with cholest-4-en-3-one-7α-ol.

As can be seen in the photograph (Fig. 38) no u.v. absorbing product was formed in the incubation involving the "supernatant" fraction of the disrupted mitochondria, and the activity for producing this substance was found to be associated with the mitochondrial debris. The activity in the debris was not increased by adding the "supernatant" fraction to it.

These results could, of course, be explained if the mitochondria had not been broken and were merely resedimented, but examination under the microscope showed definitely that the mitochondria had indeed been disrupted. These experiments were performed several times with different preparations of mitochondria.

It would appear, therefore, that firstly, disrupted mitochondria retain enzymic activity, so that on incubation with cholest-4-en-3-one-7α-ol the u.v. absorbing material (which, it has been suggested is cholest-4-en-3-one-7α,26-diol; see p. 196, is produced; and secondly that this enzymic activity is contained in the mitochondrial debris obtained after disruption.

(iii) Mitochondria were burst by osmotic means (i.e., on obtaining mitochondria they were shaken with distilled water and kept in the cold for 30 min. with frequent shaking) and the debris separated from the supernatant as before. Incubations comparing the activity of the debris obtained from sonically disrupted mitochondria with that of the debris from osmotically disrupted mitochondria were set up. The supernatants were also compared

The mitochondria used were derived from the same preparation of rat liver.

It was found that the u.v. absorbing substance was produced from cholest-4-en-3-one-7α-ol to the same extent in both the "osmotic" debris and the "sonic" debris. No activity was seen in either supernatant.

(e) Incubations with an acetone powder of mitochondria.

An acetone powder of mitochondria was prepared as described on p. 39 . This acetone powder was able to oxidise 7α-hydroxycholesterol but unable to hydroxylate it. The powder was dissolved in buffer and incubated with cholest-4-en-3-one-7α-ol, with and without NADPH. On viewing the fluorescent thin layer plates obtained from these incubations it was seen that no u.v. absorbing material was produced in either incubation. Thus it would seem that acetone treatment of the mitochondria removes any hydroxylating activity that native mitochondria contain.

(f) Effect of the hydroperoxide of linoleate

As has been discussed previously, it is possible that the hydroperoxide of linoleic acid may

play some role in hydroxylating reactions. It was therefore decided to find out whether the hydroperoxide has any effect on the hydroxylation of cholest-4-en-3-one-7α-ol.

(i) The hydroperoxide of sodium linoleate was prepared by the action of lipoxidase (p. 163). Six incubations were set up to compare the effect of the hydroperoxide on the hydroxylation of cholest-4-en-3-one-7α-ol in sonically burst mitochondria, in the debris spun down after sonication and in the supernatant thus left. Cholest-4-en-3-one-7α-ol was incubated, then, with -

a) disrupted mitochondria, with and without hydroperoxide,

b) the mitochondrial debris, with and without hydroperoxide, and

c) the supernatant with and without hydroperoxide.

The constituents of the incubation mixtures were separated as usual and the steroids run on thin layer plates which were then viewed under the u.v. light. It could be seen that including the hydroperoxide of linoleate in the incubation mixture made no difference to the formation of the polar

u.v. absorbing substance in disrupted mitochondria or mitochondrial debris. In the supernatant, as has been shown (p. 203) no hydroxylation of cholest-4-en-3-one-7α-ol takes place and this was also unaffected by the hydroperoxide.

(ii) The hydroperoxide of linoleate was prepared according to the method of Banks et al.(1959) (p. 164). The effect of linoleate hydroperoxide on the hydroxylation of cholest-4-en-3-one-7α-ol under anaerobic conditions was studied. Two incubations were set up, containing mitochondrial debris and substrate. To one incubation was added the hydroperoxide. Both incubation flasks were flushed out with nitrogen before being incubated. The steroids were extracted and run on fluorescent thin layer plates which were then viewed under the u.v. light. It was seen that the u.v. absorbing product was formed in the incubation containing no hydroperoxide but was absent from the incubation containing the hydroperoxide.

This result could be explained if an oxygen donor was in fact present in the incubation and if the linoleate hydroperoxide was inhibiting its activity. If the conditions were not truly anaerobic, however, this result does not coincide with that in (i).

C. MICROSOMES + SUPERNATANT (OR SF)

In the last Section, which dealt with the metabolism of 7α-hydroxycholesterol, it has been shown that although microsomes when incubated with 7α-hydroxycholesterol, with and without NAD, do not contain hydroxylating activity, when active supernatant or boiled deproteinised supernatant (SF) is added to the microsomes, hydroxylation can take place (see p. 176).

In this Section it has again been found that microsomes have no hydroxylating power, and it was therefore thought to be of interest to discover whether the addition of supernatant or SF might allow hydroxylation to proceed.

To carry out this investigation incubations were set up as follows:-

(a) microsomes + active supernatant;

(b) microsomes + SF

In both cases cholest-4-en-3-one-7α-ol was added as substrate. On viewing the fluorescent thin layer plates run on the ether:alcohol cuts obtained from these incubations it was found that the u.v. absorbing substance thought to be cholest-4-en-3-one-7α,26-diol was produced in both cases. (The activity, however, is not as great as that occurring in mitochondria).

These results support the observations reported on p. 176 . It seems that a thermostable factor present in the supernatant fraction of rat liver is necessary before microsomes, which are inactive alone, can hydroxylate cholest-4-en-3-one-7α-ol. This supports the evidence that the polar u.v. absorbing material obtained in incubations of microsomes + SF + NAD with 7α-hydroxycholesterol is formed by hydroxylation of cholest-4-en-3-one-7α-ol produced first by oxidation of 7α-hydroxycholesterol.

Stability of SF to pH change

An experiment was set up to discover whether the thermostable co-factor was stable to boiling with acid or alkali. Acid and alkali-treated SF was prepared as described on p. 159 and three incubations were set up, with cholest-4-en-3-one-7α-ol as substrate.

a) Microsomes + untreated SF

b) Microsomes + acid-treated SF

c) Microsomes + alkali-treated SF

The thin layer plates obtained from these incubations were viewed under the u.v. lamp and it was seen that the polar u.v. absorbing material

had been produced in incubations a) and b) only. Thus, treatment with alkali inactivates the thermostable co-factor. This is the same situation as was found to occur when mitochondria were incubated with NAD, 7α-hydroxycholesterol, acid and alkali-treated SF (p. 160).

Replacement of SF with succinate

Two incubations were set up, with cholest-4-en-3-one-7α-ol as substrate.

a) Microsomes + SF

b) Microsomes + succinate (72 μ moles)

On viewing the thin layer plates obtained from these incubations it was seen that the polar u.v. absorbing material had not been produced in incubation b). Therefore succinate cannot replace SF in this system, in its ability to promote hydroxylation of cholest-4-en-3-one-7α-ol. The results here agree with those obtained when 7α-hydroxycholesterol was incubated with mitochondria with no added NAD and addition of SF was found to stimulate hydroxylation of the substrate. Succinate could not replace SF in that case either, (p. 156).

D. SUPERNATANT FRACTION

(i) Cholest-4-en-3-one-7α-ol was incubated with "boiled" and "active" supernatant, which was prepared as described on p. 36 . The substrate was recovered by elution from the fluorescent thin layer plates obtained from such incubations and estimated either by the absorption at 242 mμ. or by radioactive means. Using unlabelled substrate and comparing the recoveries obtained from boiled and active tissue incubations, it was found that on incubation with active supernatant 12% of the added substrate had disappeared (i.e. had been metabolised). In the same way it was found on using labelled cholest-4-en-3-one-7α-ol that 11% of it had been metabolised in the active incubation.

(ii) Identification of metabolites

Thin layer plates run on the ether:alcohol cuts obtained from incubations of unlabelled substrate with active and boiled supernatants were viewed under u.v. light. No u.v. absorbing material was found to be present in the active incubation. On spraying the plates with phosphotungstic acid two products, not appearing on the plate obtained from boiled control incubation, were seen

to be formed in the active incubation. One of these products had an R_F value in the benzene:ethyl acetate:acetone system identical with that of 7α-hydroxycholesterol (i.e., 0.35). The substance also gave a blue colour with phosphotungstic acid, as does 7α-hydroxycholesterol. The other product had an R_F value of 0.43 which is identical with that given by 3α,7α-dihydroxycoprostane in the benzene:ethyl acetate:acetone system. The substance gave a yellow colour with phosphotungstic acid, as does authentic dihydroxycoprostane (see Appendix, p. 311).

(This yellow colour seems to be fairly specific, as it is given only by trihydroxycoprostane and dihydroxycoprostane out of the compounds tested.)

Thin layer plates run on the ether:alcohol cuts obtained from incubations of labelled substrate with boiled and active supernatant were segmented, eluted, and counted. No radioactivity was associated with any other part of the plate than that containing the substrate in the boiled control incubation. In the active tissue incubation, however, both the products described above were found to be labelled. About 4% of the "7α-hydroxy-

cholesterol" was formed and about 7% of the "dihydroxycoprostane".

In the other cell fractions studied, where 7α- and 7β-hydroxycholesterols were formed, they were found to be unlabelled and were therefore not formed from cholest-4-en-3-one-7α-ol. It was therefore thought to be doubtful if the "7α-hydroxycholesterol-like" product was indeed 7α-hydroxycholesterol, and on account of this uncertainty this product will henceforth be referred to as compound X.

A complete radioactive analysis was carried out on all the cuts from the columns obtained from boiled and active tissue incubations, and no further radioactive products were found in the active incubations. These observations were confirmed by spraying the plates with phosphotungstic acid and with phosphomolybdic acid.

Thus it seems that active supernatant can convert cholest-4-en-3-one-7α-ol into two further more polar compounds, one of which may be 3α,7α-dihydroxycoprostane.

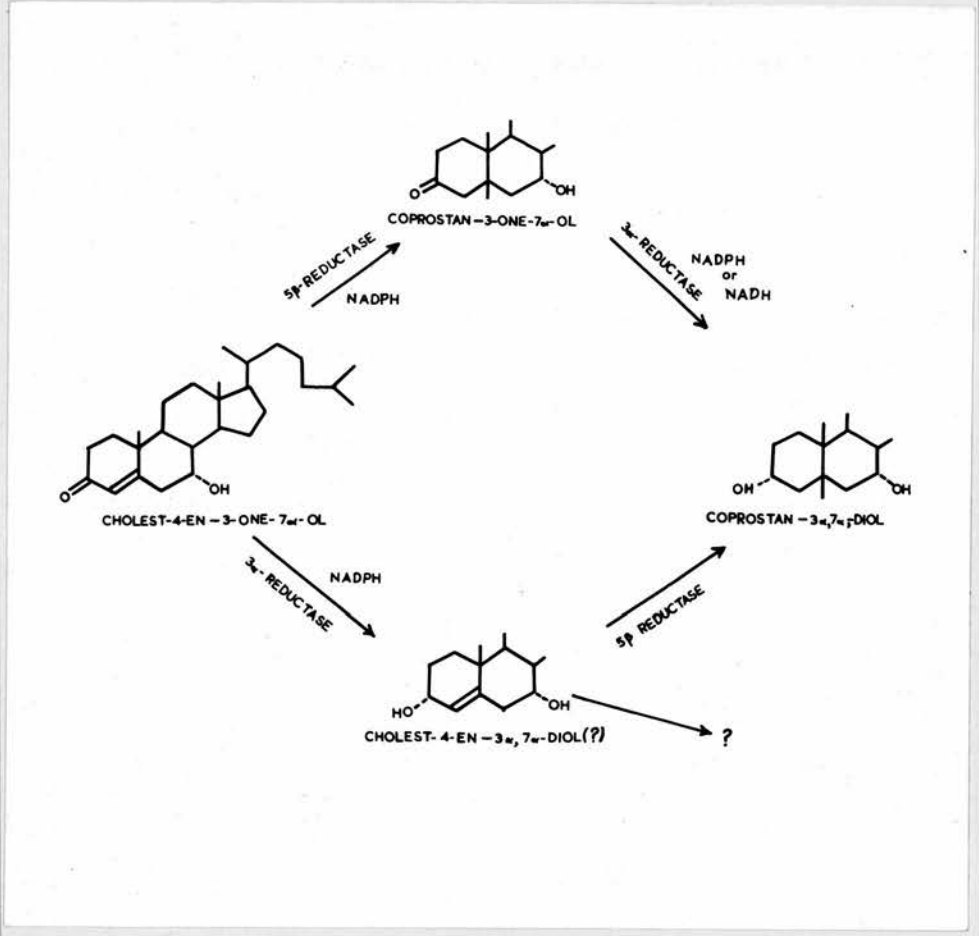

COPROSTAN-3-ONE-7α-OL
5β-REDUCTASE
NADPH
3α-REDUCTASE
NADPH
or
NADH
CHOLEST-4-EN-3-ONE-7α-OL
COPROSTAN-3α,7α-DIOL
3α-REDUCTASE
NADPH
5β REDUCTASE
CHOLEST-4-EN-3α,7α-DIOL(?)
?

Fig. 39

(iii) Purification of supernatant fraction

The reduction of cholest-4-en-3-one-7α-ol could take place by two routes (see Fig. 39). Either the double bond at 4,5 could be reduced first, forming coprostan-3-one-7α-ol which can be further reduced at the 3 position to yield 3α,7α-dihydroxycoprostane, or the 3-ketone could be attacked first forming cholest-4-en-3α,7α-diol and then the double bond could be saturated giving 3α,7α-dihydroxycoprostane. The complete reduction is an analogous reaction to the reduction of cortisone to tetrahydrocortisone. Tomkins (1956, 1957) has isolated enzymes from the supernatant fraction of rat liver which catalyse the irreversible reduction of cortisone by NADPH, firstly to the 3-keto-5β-compound, dihydrocortisone, and then to the 3α, 5β-compound, tetrahydrocortisone. It is thus possible that these "Tomkins" enzymes might be the ones found in this study which also occur in the supernatant fraction. The supernatant fraction of rat liver was therefore purified by the method due to Tomkins.

(a) Preparation of an active fraction from supernatant

The volume of the supernatant fraction,

obtained as described on p. 36, was measured and an equal volume of saturated ammonium sulphate solution was added to make the final solution 50% saturated with this substance. This mixture was stirred in the cold for 20 min. and a white precipitate was seen to be formed. This precipitate was removed by centrifugation at 10,000 x g for 15 min. in the refrigerated M.S.E centrifuge. More ammonium sulphate was added to the supernatant layer until the solution was 70% saturated with ammonium sulphate. The mixture was stirred and centrifuged as before and the precipitate was resuspended in about 10 ml. of distilled water. The solution was dialysed against 2 litres of distilled water overnight and could then be stored for several weeks at 0°C. Three fractions could thus be obtained by this procedure:-

a) the protein precipitating between 0% and 50% saturation;

b) the protein precipitating between 50% and 70% saturation; and

c) the supernatant of (b)

Tomkins found his activity in fraction (b). The activity for reducing cholest-4-en-3-one-7α-ol was also found to exist in fraction (b) using the methods described.

(b) Incubation procedure and assay system

Incubations were set up in the usual way using the following incubation mixture:-

0.5 ml. 0.05 M phosphate buffer, pH 6.4
0.1 ml. 0.0048 M NADP (4 mg./ml. of water)
0.1 ml. 0.035 M sodium glucose-6-phosphate
0.5 ml. glucose-6-phosphate dehydrogenase (equivalent to 0.5 unit, where 1 unit reduces 1 μ mole NADP)

} NADPH generating system

0.1 ml. 0.05 M magnesium sulphate
50 μg. cholest-4-en-3-one-7α-ol (in 0.025 ml. methanol)
Enzyme solution.

Incubations were carried out for 1 hr. at 37°C. in air, with shaking. The reactions were then stopped by adding 10 ml. of methanol and 20 ml. of chloroform. The mixtures were then centrifuged and the methanol layers discarded. The chloroform layers were taken to dryness, the residue dissolved in ethanol, and the amount of cholest-4-en-3-one-7α-ol disappearing during an incubation could be

estimated directly by reading the ethanolic solution at 242 mμ. No column procedure was necessary to remove interfering substances. The ethanolic solution could then be concentrated and run on fluorescent thin layer plates which could be sprayed to identify metabolites.

When labelled substrate was used the fluorescent thin layer plates were segmented, and the segments eluted with ether:alcohol (1:1) and counted, giving a complete radioactive analysis of the incubation extract.

An attempt was made to follow the reaction directly by conducting the incubation in a silica cell belonging to the u.v. recording spectrophotometer. It was hoped that by placing the cell in the spectrophotometer it would be possible to see, as the reaction proceeded, a decrease in the absorption at 242 mμ. as the substrate was metabolised and a concomitant decrease in absorption at 340 mμ. as the NADPH was used up.

NADPH was therefore generated first by incubating the constituents of the NADPH generating system in buffer for 30 min. and stopping the reaction by boiling. The enzyme and substrate were then added to the generated NADPH and the solution

was put into the silica cell of the spectrophotometer. However, on placing the cell into the spectrophotometer and reading against a blank containing NADPH and enzyme, it was found to be impossible to balance the machine in the 200 - 270 mμ. wave-length region because of the amount of protein present. It was possible, however, to balance the machine in the 340 mμ region and a slight decrease in absorption at 340 mμ. could be seen with increasing time.

Thus, it seems that it is impossible to follow the reaction directly with this partially purified enzyme. Further purification of the enzyme might make this technique a possible one to use, as less protein would be required for activity.

(c) Identification of metabolites

Thin layer plates run on the chloroform extracts from incubations of cholest-4-en-3-one-7α-ol with this partially purified enzyme were viewed under the u.v. light and then sprayed with phosphotungstic acid, (Fig. 49) Comparison of an incubation stopped at zero time with one stopped after 1 hr. showed that the two products formed by incubations with unpurified supernatant were produced in the latter (p. 211).

Using labelled substrate and segmenting, eluting, and counting the thin layer plate showed that these two products were labelled, and that no further products were formed. It was found that about 10% of compound X (p. 213) and 45% of the "dihydroxycoprostane" were formed.

It is therefore clear that the ammonium sulphate treatment brought about considerable purification of the enzyme.

(i) Identification of compound X

As has been discussed on p. 214 , cholest-4-en-3-one-7α-ol could be reduced in two ways. It is possible, although not very likely, that the 3-ketone group could be reduced to yield a 3β-hydroxyl group rather than a 3α-hydroxyl group, or that on reduction of the double bond the hydrogen might occupy the 5α-position. Thus, the reduction of the substrate could give rise to the following compounds:-

(a) cholest-4-en-3β,7α-diol,

(b) cholest-4-en-3α,7α-diol,

(c) coprostan-3-one-7α-ol,

(d) cholestan-3-one-7α-ol,

(e) 3α,7α-dihydroxycoprostane,

(f) 3β,7α-dihydroxycoprostane,

(g) 3α,7α-dihydroxycholestane,

(h) 3β,7α-dihydroxycholestane,

(i) 7α-hydroxycholesterol.

By a study of the mobility of compound X and of its properties many of these possibilities can be eliminated. Thus, (c) and (d) can be eliminated because they would be less polar than the substrate, and also would not give a blue colour with phosphotungstic acid. Compounds (e), (f), (g) and (h) would all be less polar than compound X and again would not give the blue colour, so they too can be eliminated. However, compounds (a) and (b) and also (i) all have properties similar to those of compound X.

To discover whether compound X was 7α-hydroxycholesterol, or either cholest-4-en-3α,7α-diol, or the 3β-epimer, the following experiment was carried out.

Oxidation of compound X with manganese dioxide

If compound X were 7α-hydroxycholesterol it would yield 7-ketocholesterol when oxidised with manganese dioxide. If, on the other hand compound X were either cholest-4-en-3α,7α-diol or the 3β-epimer it would give cholest-4-en-3-one-7α-ol (see Fig. 41).

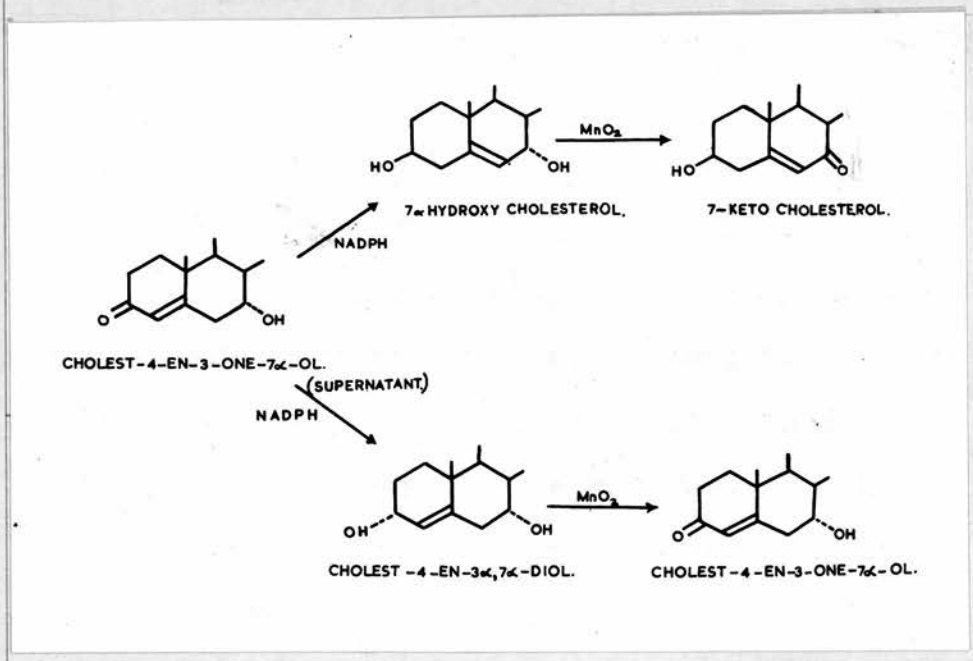


FIG. 41

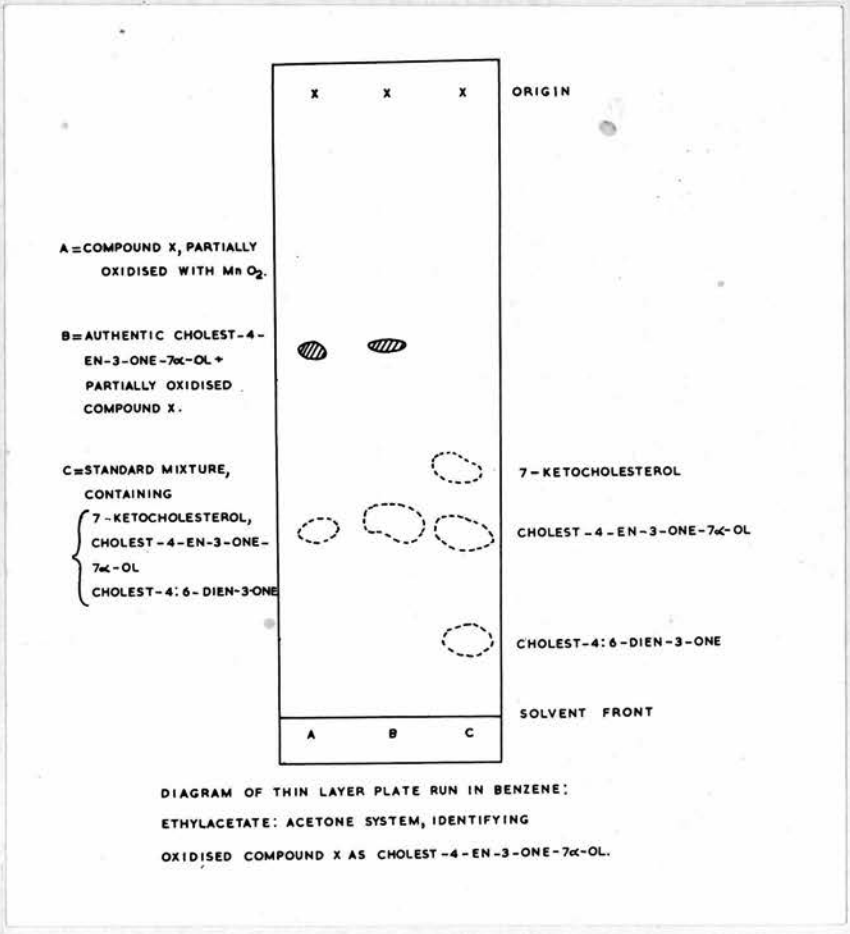


DIAGRAM OF THIN LAYER PLATE RUN IN BENZENE: ETHYLACETATE: ACETONE SYSTEM, IDENTIFYING OXIDISED COMPOUND X AS CHOLEST-4-EN-3-ONE-7α-OL.

FIG. 42

A thin layer plate was run on the extract from an incubation with labelled substrate and, without spraying, the section of the plate containing compound X was eluted with chloroform and the silicic acid removed by centrifugation. The chloroform solution was shaken with manganese dioxide for 6 hr. and then left at room temperature for 18 hr. The solution was then filtered, concentrated, and re-run on a fluorescent thin layer plate. On the same plate was run a standard solution containing both cholest-4-en-3-one-7α-ol and 7-ketocholesterol. It was found that oxidised compound X was u.v. absorbing and had the same mobility as cholest-4-en-3-one-7α-ol. The oxidised compound X was eluted from the plate and found to be labelled. The experiment was repeated but after oxidation the chloroform solution was halved. One half of the solution was run on a fluorescent plate as before and the other half was mixed with authentic cholest-4-en-3-one-7α-ol and also run on the plate. The standard solution was also included on the same plate (see Fig. 42). On viewing the plate after running it was seen that oxidised compound X did not separate from authentic cholest-4-en-3-one-7α-ol, which was found to be labelled on elution from the plate.

These results show that compound X, on oxidation with manganese dioxide gives rise to cholest-4-en-3-one-7α-ol and therefore compound X cannot be 7α-hydroxycholesterol but must be either cholest-4-en-3α,7α-diol or cholest-4-en3β,7α-diol.

Re-incubation of compound X with supernatant enzyme

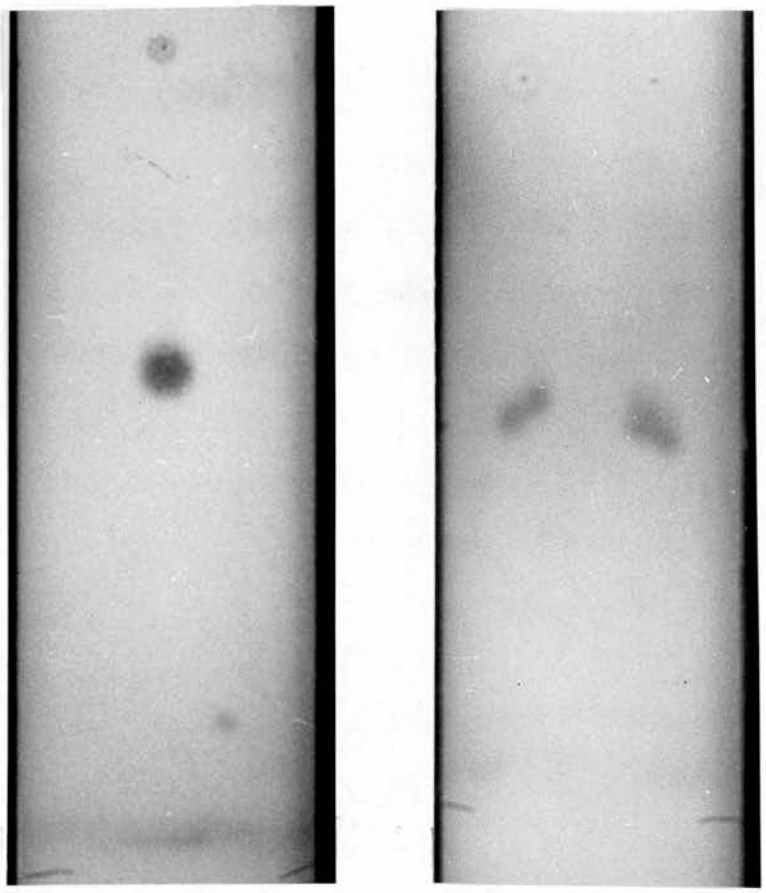
In order to discover if compound X is an intermediate between cholest-4-en-3-one-7α-ol and the compound thought to be 3α,7α-dihydroxycoprostane (see p. 219), compound X was isolated from an incubation with cholest-4-en-3-one-7α-ol and supernatant enzyme. This isolation was carried out by eluting compound X from an unsprayed thin layer plate. Before incubating compound X with enzyme, a small amount was run on another plate and sprayed, to ensure that no 3α,7α-dihydroxy-coprostane which had also been formed in the first incubation had also been eluted. Compound X was then incubated with supernatant enzyme and NADPH generator for an hour. The steroids were extracted as usual and the chloroform extract was concentrated and run on a thin layer plate. On spraying the plate with phosphotungstic acid and

phosphomolybdic acid it could be seen that a very small amount of 3α,7α-dihydroxycoprostane had been formed. On repeating the experiment and re-incubating compound X without NADPH, it was seen that no 3α,7α-dihydroxycoprostane was formed.

Thus, it would seem that compound X is an intermediate between cholest-4-en-3-one-7α-ol and 3α,7α-dihydroxycoprostane, although it does not seem to be very readily converted into the latter substance. However, this would indicate that compound X is probably cholest-4-en-3α,7α-diol and not the 3β-epimer.

Experiments with authentic cholest-4-en-3α, 7α-diol

(i) Cholest-4-en-3α,7α-diol was prepared by Mr. Naqui by the method described in the Appendix p. 305. This substance was incubated with the supernatant enzyme with and without NADPH. The steroids were extracted as usual and run on thin layer plates which were sprayed to identify metabolites. There appeared to be very little conversion of cholest-4-en-3α,7α-diol to dihydroxycoprostane in the presence of NADPH. Thus cholest-4-en-3α,7α-diol is not readily converted by this enzyme preparation to dihydroxycoprostane.



a) b)

1 2

Fig. 43.

Photographs of thin layer plates, sprayed with phosphomolybdic acid.

1. 3α,7α, dihydroxycoprostane isolated from an incubation of cholest-4-en-3-one-7α-ol with supernatant enzyme, mixed with authentic dihydroxy-coprostane and re-run in benzene:ethyl acetate: acetone.

2. a) cholest-4-en-3α,7α-diol isolated as in 1.
   b) cholest-4-en-3α,7α-diol isolated as in 1, mixed with authentic cholest-4-en-3α, 7α-diol and re-run in benzene:ethyl acetate:acetone.

(ii) Compound X was isolated from an incubation as described above (p.222) and re-run on a thin layer plate together with authentic cholest-4-en-3α,7α-diol. Compound X did not separate from the standard in the benzene:ethyl acetate:acetone system (Fig. 43). Cholest-4-en-3α,7α-diol does separate from the 3β-epimer in this sytem, the 3β-epimer being less polar than the 3α,epimer.

These results show, then, that compound X, which is formed from cholest-4-en-3-one-7α-ol when incubated with a partially purified enzyme from the supernatant fraction of rat liver, is probably cholest-4-en-3α,7α-diol, although there is a slight possibility that it could be the 3β-epimer. Compound X is presumably formed by reduction of cholest-4-en-3-one-7α-ol with NADPH as co-factor and on incubation with the enzyme forms 3α,7α-dihydroxycoprostane, although not very readily. Thus, cholest-4-en-3α,7α-diol is a possible intermediate on the pathway from cholesterol to bile acids.

(ii) <u>Identification of "dihydroxycoprostane"</u>

The other product, less polar than compound X, formed when cholest-4-en-3-one-7α-ol is incubated

with the purified supernatant enzyme had an R_F value of 0.43 in the benzene:ethyl acetate:acetone system, and 0.06 in the benzene:dioxane system. These R_F values are identical with those given by authentic 3α,7α-dihydroxycoprostane in the above systems. The product gives a yellow colour with phosphotungstic acid as does 3α,7α-dihydroxycoprostane, and this yellow colour seems to be fairly specific.

A thin layer plate obtained from an incubation of cholest-4-en-3-one-7α-ol with the enzyme was not sprayed but the portion containing the product was scraped off and eluted. The eluate was taken to dryness and concentrated sulphuric acid was added to the residue. A yellow colour was seen to develop and after one hr. the u.v. absorption spectrum of the solution was plotted. The spectrum was found to be similar to that given by an authentic sample of 3α,7α-dihydroxycoprostane, i.e. a peak at 312 mμ. was obtained. This reaction is not very sensitive, about 25 μg. of dihydroxycoprostane being required to give an optical density of 0.1 at 312 mμ. A tracing of the two spectra is given in Fig. 44.

The product was isolated as above from an incubation with labelled cholest-4-en-3-one-7α-ol and mixed with genuine 3α,7α-dihydroxycoprostane. The mixture was run in three different solvent systems on thin layer plates, viz., benzene:ethyl acetate (2:1), benzene:dioxane (19:1), and benzene:ethyl acetate:acetone (10:5:3). On spraying the plates it was seen that the product had not separated from 3α,7α-dihydroxycoprostane in any of the systems (see Fig. 43). Similar plates were run, but not sprayed. These were segmented, eluted and counted, and it was found that the radioactivity was concentrated on the plate in the section where the dihydroxycoprostane should be located.

The product was isolated from several incubations as above, and the infra-red spectrum of the product was plotted. This was compared with the infra-red spectrum of authentic dihydroxycoprostane. The spectrum of the product showed a peak in the hydroxyl region, and was similar to the spectrum of the pure substance. There was not really enough of the product to attempt to plot the infra-red spectum and the region from about 10 - 15 microns wave-length, which is different

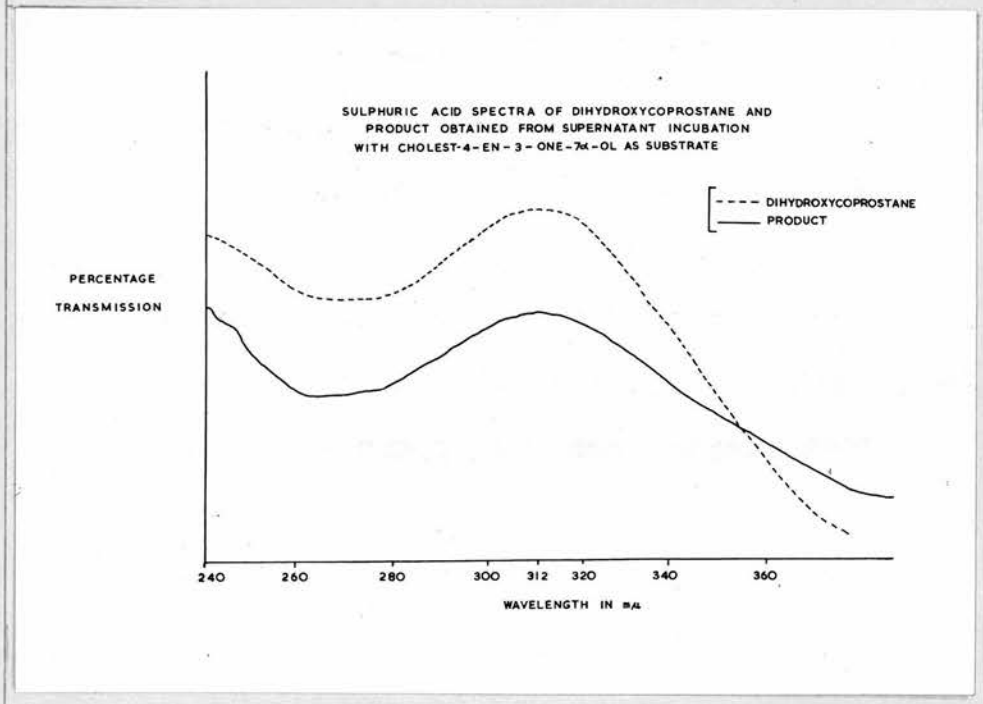


Figure 44

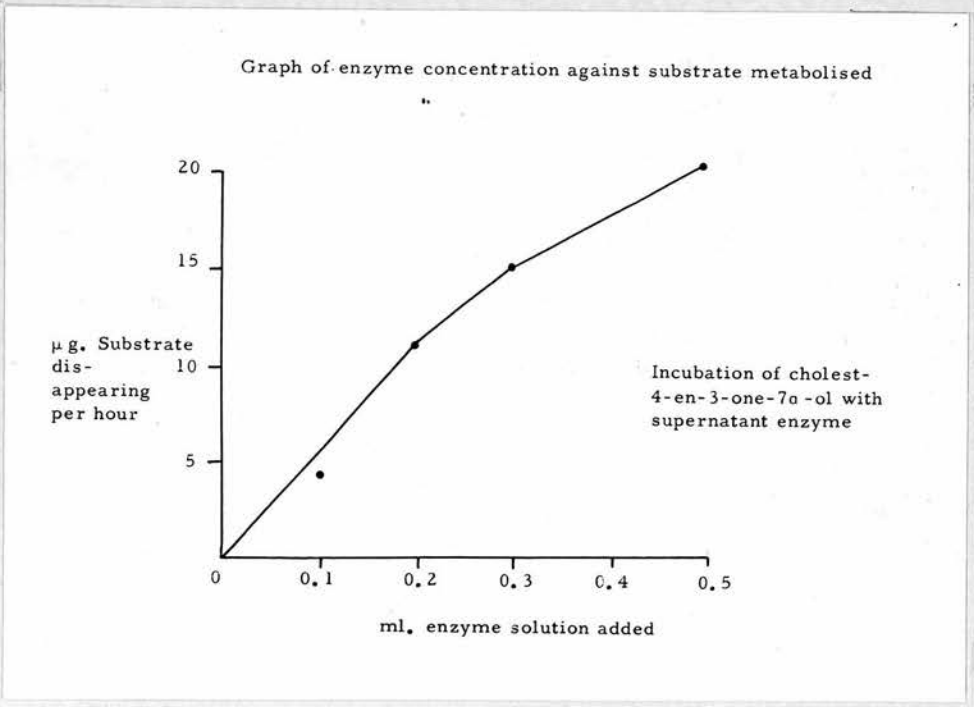


Figure 45

for every compound ("finger-print region") did not show up any characteristic peaks.

The cumulative evidence produced here indicates that one of the products obtained by incubating cholest-4-en-3-one-7α-ol with a partially purified enzyme from the supernatant fraction of rat liver is 3α,7α-dihydroxycoprostane, which has been shown by Bergstrom and Lindstedt (1956) to be converted to bile acids in the fistula rat.

(c) Metabolism of cholest-4-en-3-one-7α-ol studied in the purified supernatant fraction under various conditons

(i) Variation in enzyme concentration

Four incubations were set up, each containing the same amount of NADPH generating system and substrate (40 µg.) (see incubation mixture, p. 188) but each containing a different amount of enzyme, varying from 0.1 ml. to 0.5 ml. enzyme. The steroids were extracted from the incubation mixture as described (p. 50) and the residues from the chloroform extractions were read in ethanol at 242 mµ. The amount of substrate disappearing in each case could then be calculated, and is given in Table 34. A graph was drawn of amount of enzyme added against amount of substrate

disappearing, and showed that as the enzyme concentration increased the amount of substrate which disappeared also increased (Fig. 45).

TABLE 34

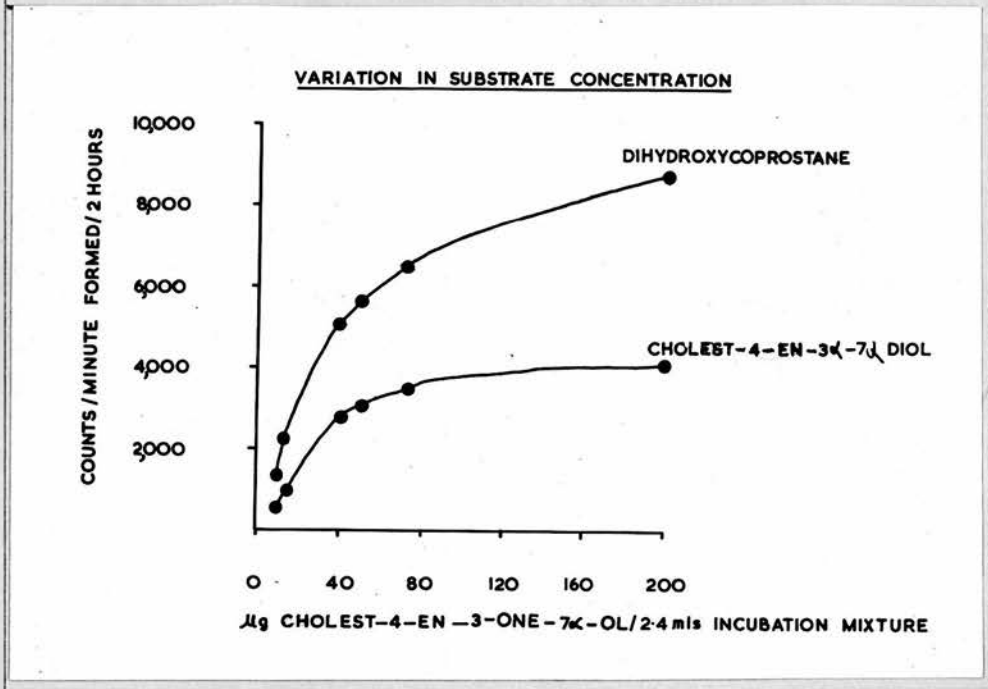
ml. enzyme added to incubation		Substrate recovered: Optical density at 242 mμ.	μg.	Substrate disappearing
a)	0.1	0.500	35.7	4.3
b)	0.2	0.400	29	11
c)	0.3	0.355	25.3	14.7
d)	0.5	0.280	20	20

40 μg. substrate added to each incubation (equivalent to an optical density of 0.57)

After reading the ethanolic solutions at 242 mμ. the solutions were concentrated and run on thin layer plates, which were then sprayed with phosphotungstic acid. It was found that as the enzyme concentration increased, the amounts of both products also increased.

(ii) Variation in substrate concentration

In the following experiments, labelled cholest-4-en-3-one-7α-ol was used as the substrate in incubations with the enzyme. A measure of the


VARIATION IN SUBSTRATE CONCENTRATION
COUNTS / MINUTE FORMED / 2 HOURS
10,000
8,000
6,000
4,000
2,000
DIHYDROXYCOPROSTANE
CHOLEST-4-EN-3α-7α DIOL
0
40
80
120
160
200
μg CHOLEST-4-EN-3-ONE-7α-OL/2·4 mls INCUBATION MIXTURE

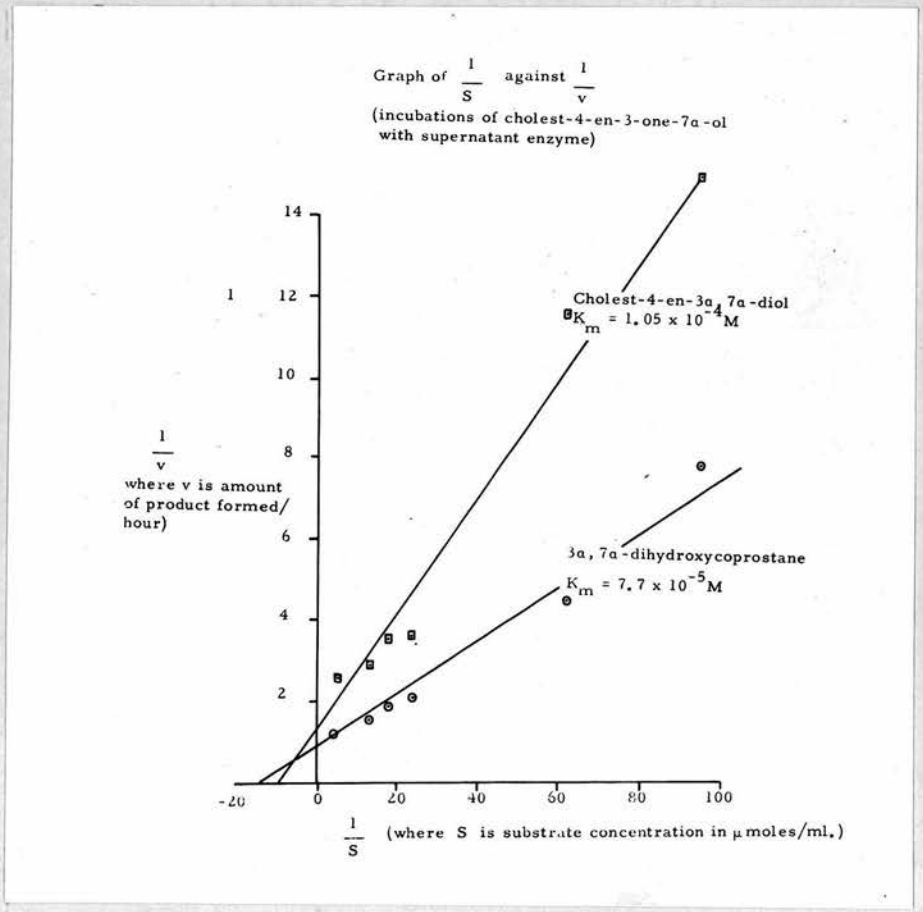
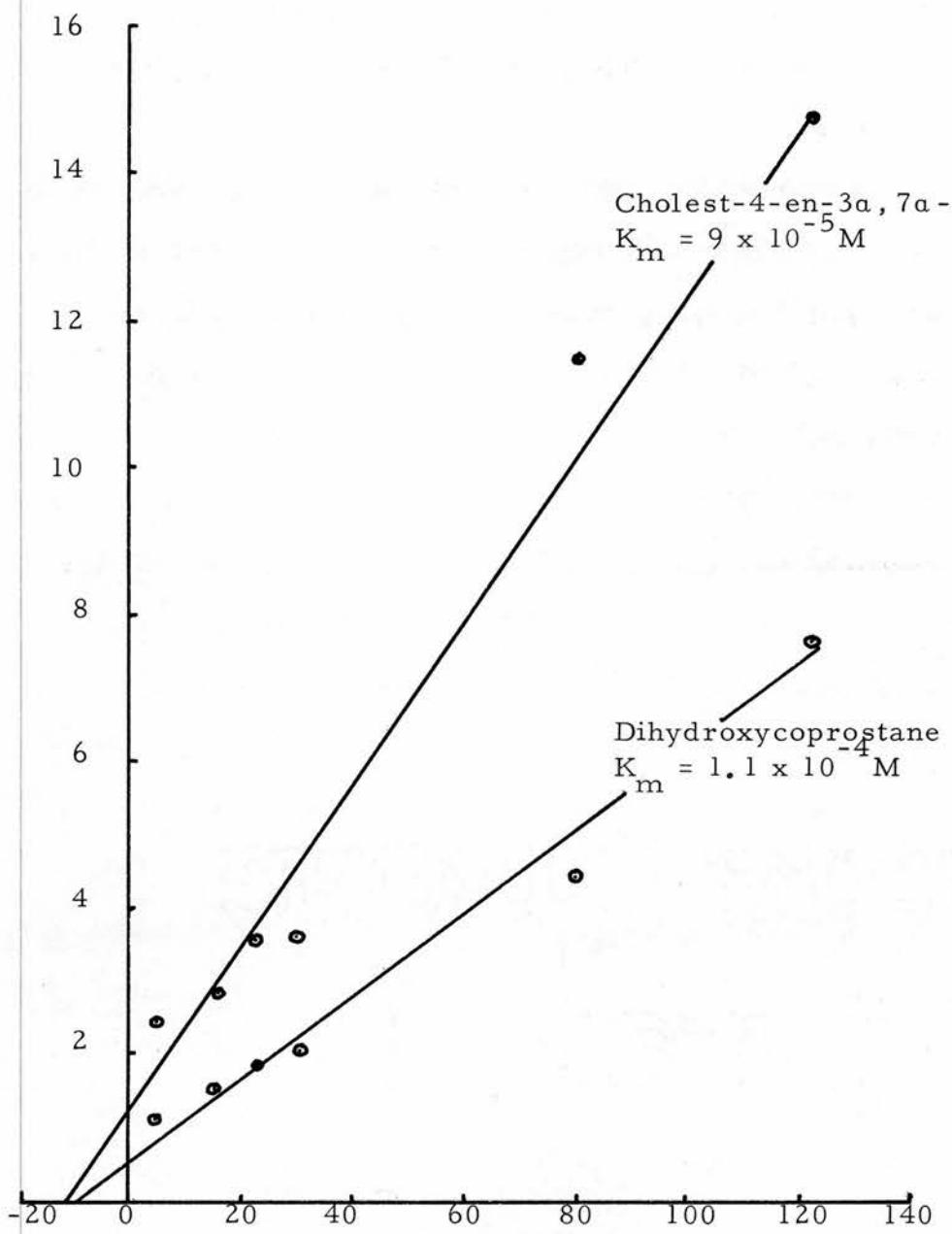

Graph of 1/S against 1/v
(incubations of cholest-4-en-3-one-7α-ol with supernatant enzyme)
1/v
where v is amount of product formed/ hour)
14
12
10
8
6
4
2
Cholest-4-en-3α, 7α-diol
Km = 1.05 x 10-4 M
3α, 7α-dihydroxycoprostane
Km = 7.7 x 10-5 M
-20
0
20
40
60
80
100
1/S (where S is substrate concentration in μ moles/ml.)

Figure 46

Graph of $\frac{1}{S}$ against $\frac{1}{v}$ (incubations of cholest-4-en-3-one-7α-ol with supernatant enzyme).



$\frac{1}{v}$ (where v is amount of product formed/hour)

$\frac{1}{S}$ (where S is substrate concentration in μ moles/ml.)

Figure 46(a)

The initial substrate concentration is corrected in this figure, for the amount of substrate disappearing, using a factor $\frac{\log\left(\frac{1}{1-f}\right)}{f}$, where f is the fraction of the initial substrate disappearing (Fisher, R. B. and Parsons, D. S., J. Physiol., 119, 210 (1953)).

metabolism of the substrate was obtained by isolating and counting the two products, cholest-4-en-3α,7α-diol and 3α,7α-dihydroxycoprostane formed.

Incubations were set up containing NADPH-generating system, 1 ml. enzyme solution and amounts of labelled substrate varying from 10 μg. to 200 μg. After incubation, the two labelled products were isolated from thin layer plates by elution and were estimated by radioactive methods. The results are shown in Table 35. Graphs of substrate concentration against (a) the amount of dihydroxycoprostane formed per hr. (in c/m) and (b) the amount of cholest-4-en-3α,7α-diol formed per hr. (in c/m) were drawn, (Fig. 46).

The results show that the amounts of both products formed increase with increasing substrate concentration sharply up to about 50 μg. substrate. Further increase in substrate concentration does not give rise to such a sharp increase in the amount of products formed, the curve being thus typical of an enzymic reaction. A graph was constructed of the reciprocal of the substrate concentration against the reciprocal of the reaction velocity (i.e., the amount of product formed per

TABLE 35

Partially Purified Supernatant Fraction

Variation in substrate concentration
(specific activity of substrate = 500 c/m/μg.)

Cholest-4-en-3-one-7α-ol added μg.	Dihydroxycoprostane formed c/m/2 hr.	Cholest-4-en-3α,7α-diol formed c/m/2 hr.
10	1290	675
15	2127	870
40	4900	2772
50	5470	2800
72	6384	3472
200	8700	4060

Substrate added μ Moles/ml (s)	$\frac{1}{s}$	Dihydroxy-coprostane $\frac{1}{v}$ (x 10^{-4})	Cholest-4-en-3α,7α-diol $\frac{1}{v}$ (x 10^{-4})
0.0104	96	7.75	14.80
0.0160	62.5	4.40	11.50
0.0417	24	2.04	3.60
0.052	19.2	1.83	3.57
0.073	13.7	1.57	2.88
0.208	4.8	1.15	2.46

(where v, the reaction velocity, is equal to the amount of product formed, in c/m/2 hr.).

hr.) for each product and both plates were found to be straight lines. From the intercepts on the horizontal axis the K_m values for each reaction could be calculated (Fig. 46).

(iii) Co-factor requirements

(a) Incubations with 1 ml. enzyme and 50 µg. unlabelled substrate were set up to compare the effect of adding no co-enzyme, NADPH or NADH on the formation of the two products. Thin layer plates obtained from such incubations were sprayed firstly with phosphotungstic acid and then with phosphomolybdic acid.

No products at all could be detected when either NADH (equivalent to the amount of NADPH which is generated by the glucose-6-phosphate system normally used) or no co-enzyme was added to the incubation. The two products, cholest-4-en-3α,7α-diol and dihydroxycoprostane were, however, seen to be formed when NADPH (in the form of the generating system) was added.

These results show that the reduction of cholest-4-en-3-one-7α-ol requires NADPH as co-enzyme, and that the NADPH cannot be replaced by NADH.

(b) Variation in NADPH concentration

In order to know the exact amount of NADPH added to the incubation mixtures, the NADPH was generated before adding to the mixtures, instead of allowing it to be produced in the incubating mixtures themselves.

A mixture containing:-

0.2 ml. NADPH (0.0048 M)

0.2 ml. glucose-6-phosphate (0.035 M)

0.2 ml. magnesium sulphate (0.05 M)

0.1 ml. glucose-6-phosphate dehydrogenase (equivalent to 1 unit, where 1 unit reduces 1 μ mole NADP)

2.3 ml. phosphate buffer, pH 6.4

was incubated for 30 min. at 37°. The absorption spectrum of the solution was then plotted, and from the optical density reading at 340 mμ. it was calculated that 0.85 μ mole of NADPH had been formed in 3 ml. This solution was boiled to stop any reaction and was then used to add known amounts of NADPH to the incubation mixtures.

Incubations were set up, each containing the same amount of labelled substrate and enzyme (1 ml.) but each with a different amount of NADPH, varying from zero to 0.5 μ moles. The thin layer plates obtained from these incubations were segmented and

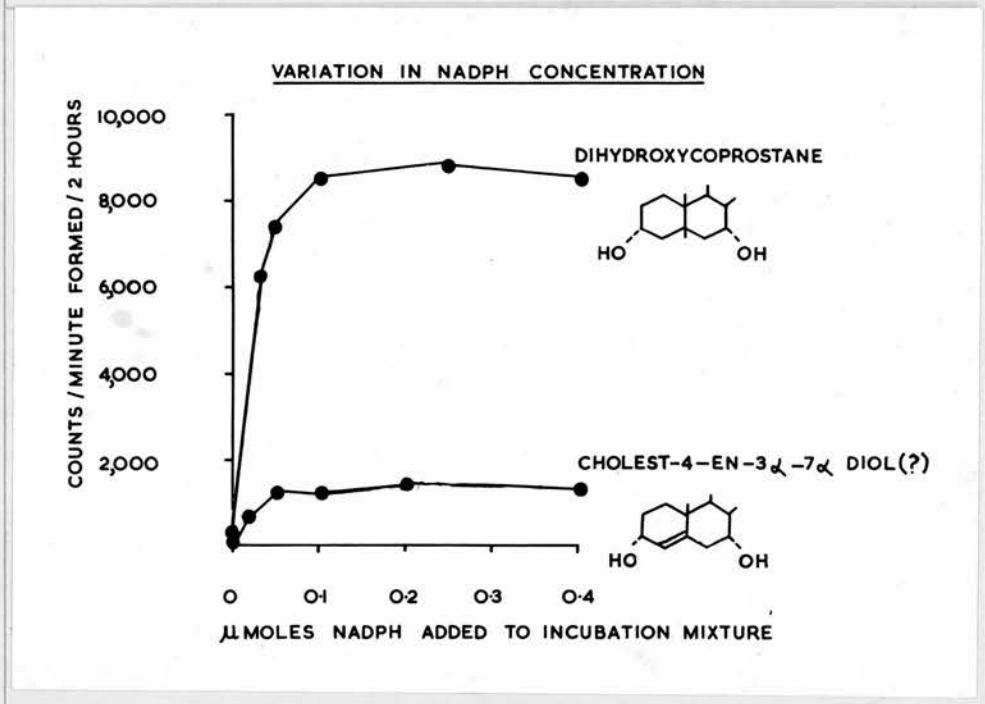

VARIATION IN NADPH CONCENTRATION
COUNTS / MINUTE FORMED / 2 HOURS
10,000
8,000
6,000
4,000
2,000
DIHYDROXYCOPROSTANE
HO
OH
CHOLEST-4-EN-3α-7α DIOL(?)
HO
OH
0
0·1
0·2
0·3
0·4
μ MOLES NADPH ADDED TO INCUBATION MIXTURE

Fig. 47

eluted and the amounts of both products formed and the amount of substrate disappearing were estimated by radioactive means. The results are given in Table 36 .

Graphs were drawn of NADPH concentration against (a) amount of substrate disappearing in 2 hr., (b) the amount of dihydroxycoprostane in 2 hr. and (c) the amount of cholest-4-en-3α,7α-diol formed in 2 hr. It can be seen from these graphs (Fig. 47) that the amount of substrate disappearing with concomitant rise in products formed increases as NADPH concentration increases, sharply up to about 0.1 μ mole NADPH. Concentrations of NADPH greater than 0.1 μ mole/2.4 ml. gave no further increase in products formed or substrate disappearing. Thus the enzyme would appear to be saturated at about 0.1 μ mole NADPH/2.4 ml. It would seem, therefore, that the formation of both cholest-4-en-3α,7α-diol and dihydroxycoprostane from cholest-4-en-3-one-7α-ol is dependent on the NADPH concentration in the medium.

(c) Cholest-4-en-3-one studied as a possible inhibitor

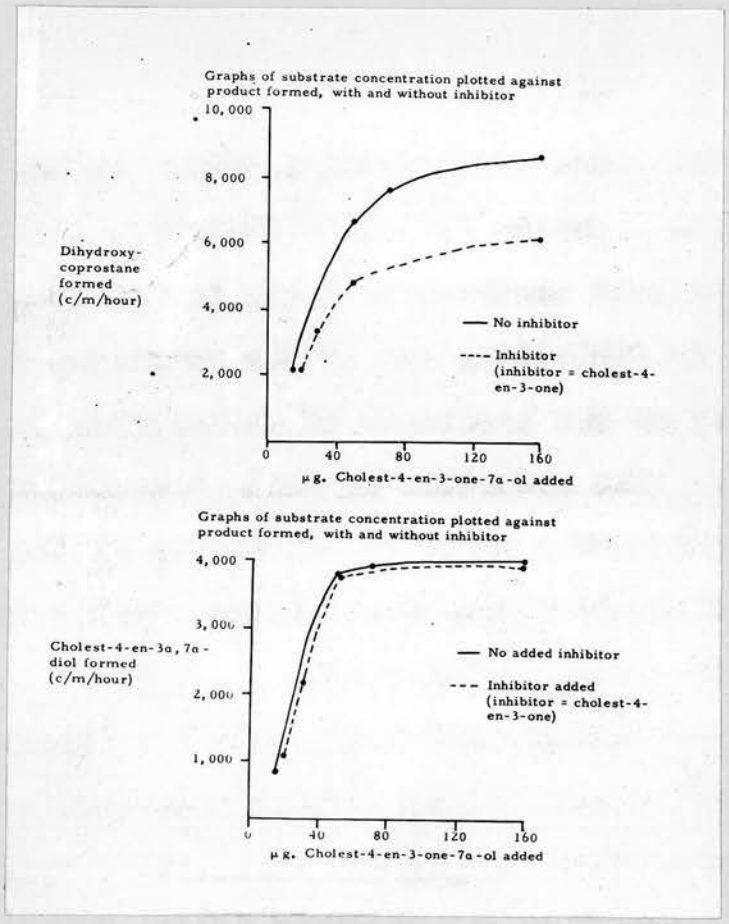
As cholest-4-en-3-one is such a toxic substance when given to an animal in vivo

TABLE 36

Partially Purified Supernatant Fraction

(Concentration of NADPH varied
(50 µg. (25,000c/m) cholest-4-en-3-one-7α-ol added as substrate

µ Moles NADPH added	Cholest-4-en-3α,7α-diol formed (c/m/2 hr.)	Dihydroxycoprostane formed (c/m/2 hr.)	Substrate Disappearing (c/m/2 hr.)
0	0	300	400
0.025	625	6298	6900
0.05	1218	7366	8580
0.1	1215	8579	9800
0.25	1500	8779	10,400
0.4	1263	8410	10,580


Graphs of substrate concentration plotted against product formed, with and without inhibitor
10,000
8,000
6,000
4,000
2,000
Dihydroxy-coprostane formed (c/m/hour)
No inhibitor
Inhibitor (inhibitor = cholest-4-en-3-one)
0
40
80
120
160
μg. Cholest-4-en-3-one-7α-ol added
Graphs of substrate concentration plotted against product formed, with and without inhibitor
4,000
3,000
2,000
1,000
Cholest-4-en-3α, 7α-diol formed (c/m/hour)
No added inhibitor
Inhibitor added (inhibitor = cholest-4-en-3-one)
0
40
80
120
160
μg. Cholest-4-en-3-one-7α-ol added

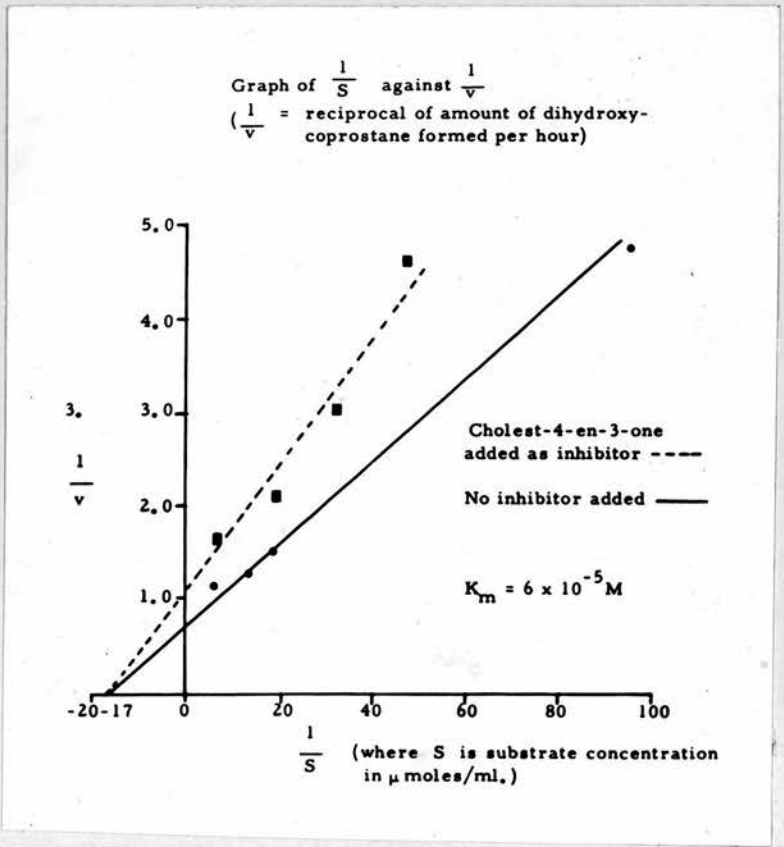

Graph of 1/S against 1/v
(1/v = reciprocal of amount of dihydroxy-coprostane formed per hour)
5.0
4.0
3.0
2.0
1.0
3.
1/v
Cholest-4-en-3-one added as inhibitor ----
No inhibitor added ——
$K_m = 6 \times 10^{-5}$M
-20-17
0
20
40
60
80
100
1/S (where S is substrate concentration in μ moles/ml.)

Fig. 48

(Steinberg and Frederickson, 1956; Tomkins, Nichols, Chapman, Hotta and Chaikoff, 1957) it was wondered whether, in fact, the cholest-4-en-3-one is inhibiting one of the reactions on the pathway of the breakdown of cholesterol to bile acids. The reduction of cholest-4-en-3-one-7α-ol might be the reaction inhibited, as the structure of cholest-4-en-3-one is so similar to that of cholest-4-en-3-one-7α-ol.

The possible inhibitory effect of cholest-4-en-3-one was tested by setting up incubations containing a fixed amount (50 μg.) with varying amounts of cholest-4-en-3-one-7α-ol. The two reduction products of the substrate were isolated from thin layer plates and estimated by radioactive means. These incubation results were compared with results obtained from incubations with varying amounts of substrate but containing no inhibitor. These results are shown in Table 37 Graphs were drawn of substrate concentration against (a) the amount of dihydroxycoprostane formed and (b) the amount of cholest-4-en-3α,7α-diol. These graphs were compared with the corresponding ones obtained with added inhibitor (Fig. 48)

TABLE 37

Partially Purified Supernatant Fraction

(a) Variation in substrate concentration

(b) Variation in substrate concentration, with fixed inhibitor concentration. (Inhibitor = cholest-4-en-3-one)
Specific activity of substrate = 500 c/m/µg.

	Cholest-4-en-3-one -7α-ol added µg.	Inhibitor added µg.	Dihydroxycoprostane formed (c/m/hr.)	Cholest-4-en-3α, 7α-diol formed (c/m/hr.)
a)	15	-	2100	800
	50	-	6517	3825
	70	-	7553	3913
	160	-	8600	4000
b)	20	50	2133	1060
	30	50	3280	2146
	50	50	4695	3705
	160	50	6020	3900

Substrate added μ Moles/ml. (s)	$\frac{1}{s}$	Dihydroxycoprostane $\frac{1}{v}$ (x 10^{-4})	Cholest-4-en-3α,7α-diol $\frac{1}{v}$ (x 10^{-4})
a) **No inhibitor**			
0.0104	96	4.76	12.5
0.052	19.2	1.53	2.61
0.073	13.7	1.32	2.56
0.166	6.02	1.16	2.50
b) **Inhibitor**			
0.021	47.6	4.69	9.34
0.031	32.3	3.05	4.65
0.052	19.2	2.13	2.69
0.166	6.02	1.66	2.56

(where v, the reaction velocity, = amount of product formed in c/m/hr.)

It can be seen that adding cholest-4-en-3-one to incubations with cholest-4-en-3-one-7α-ol gives rise to less dihydroxycoprostane than is formed without inhibitor. However, inhibitor does not seem to affect the formation of cholest-4-en-3α,7α-diol. Plotting the reciprocal of the substrate concentration against the reciprocal of the reaction velocity shows that the inhibitory action of cholest-4-en-3-one on the formation of dihydroxycoprostane is of a non-competitive nature, as the K_m value of the inhibited reaction is the same as that of the uninhibited reaction (Fig. 48).

The fact that the formation of cholest-4-en-3α,7α-diol is unaffected by inhibitor, whereas the formation of dihydroxycoprostane is affected is not in accord with the theory that cholest-4-en-3-one-7α-ol is reduced firstly to cholest-4-en-3α,7α-diol which is further reduced to dihydroxycoprostane. One explanation of these results is that dihydroxycoprostane may be formed by another route from cholest-4-en-3-one-7α-ol in this enzyme preparation (see p. 214).

(d) Activity of enzyme towards several Δ_4-3-ketones

As this partially purified enzyme is presumably the same preparation as that described by Tomkins, (1956, 1957) and should therefore be capable of reducing cortisone, it was decided to test the specificity of the preparation by incubating several Δ_4-3-ketones with it.

Solutions containing approximately 50 µg. in 0.025 ml. of methanol of the following were prepared.

(a) progesterone,

(b) cortisol acetate,

(c) cortisone acetate,

(d) corticosterone acetate,

(e) cholest-4-en-3-one,

(f) androst-4-en-3,17-dione,

(g) cholest-4-en-3-one-7α-ol.

Incubations were set up containing NADPH-generator, 1 ml. enzyme and 50 µg. of each substrate. Two incubations were set up for each substrate, one of which was stopped at zero time to act as a control, while the other was allowed to incubate for 2 hr. The incubations were extracted as usual and the residues from the extractions were

dissolved in ethanol and their absorption spectra plotted. The absorption spectra of standard solutions of each steroid were also plotted.

The ethanolic solutions were then concentrated and run on thin layer plates which were sprayed to identify products, and photographed. The number of μ moles of each steroid metabolised are shown in Table 38 and it can be seen, firstly that the enzyme is very active towards C_{21}-steroids, such as androst-4-en:3:17-dione; secondly that cholest-4-en-3-one, used in the inhibitor studies is also metabolised very slightly but that cholest-4-en-3-one-7α-ol is more readily reduced. Other experiments comparing the reductive activity of the enzyme towards cholest-4-en-3-one and cholest-4-en-3-one-7α-ol showed that the former substrate was not metabolised at all. The fact that cholest-4-en-3-one may be a substrate for this enzyme preparation complicates the conclusions that can be drawn from inhibitor studies.

Metabolism of coprostan-3-one-7α-ol

There are two possible routes for the formation of 3α,7α-dihydroxycoprostane from cholest-4-en-3-one-7α-ol, as has been discussed on p. 214 and as is shown in Fig. 39. It was thought that

TABLE 38

Activity of Supernatant Enzyme Towards Various Substrates

Substrate	Optical Density at 240 mμ.		Difference in optical density at 240 mμ.	μ moles substrate metabolised
	Control	Active		
1. Cholest-4-en-3-one-7α-ol	0.72	0.32	0.40	0.08
2. Cholest-4-en-3-one	0.62	0.544	0.076	0.014
3. Cortisol Acetate	0.493	0.061	0.432	0.081
4. Cortisone Acetate	0.608	0.065	0.543	0.102
5. Corticosterone Acetate	0.788	0.19	0.598	0.112
6. Progesterone	1.12	0.37	0.75	0.14
7. Androst-4-en-3:17-dione	1.04	0.105	0.935	0.175

as cholest-4-en-3α,7α-diol and 3α,7α-dihydroxy-coprostane were obtained as reduction products of cholest-4-en-3-one-7α-ol by the supernatant enzyme, the route for the formation of dihydroxy-coprostane must lie via cholest-4-en3α,7α-diol. However, the facts that (a) authentic cholest-4-en-3α,7α-diol was not readily converted into dihydroxycoprostane by this enzyme, and (b) the inhibition studies using cholest-4-en-3-one indicated that only the formation of dihydroxy-coprostane was affected by inhibitor, are not in accord with this theory. Thus, it seemed advantageous to discover whether coprostan-3-one-7α-ol was a substrate for this enzyme and could be converted into dihydroxycoprostane.

(The synthesis of coprostan-3-one-7α-ol is described in the Appendix, p. 206).

Incubations of coprostan-3-one-7α-ol with supernatant enzyme

(a) Incubations were set up containing NADPH-generating system and enzyme to compare cholest-4-en-3-one-7α-ol, coprostan-3-one-7α-ol and cholest-4-en-3α,7α-diol as substrates for the enzyme. After incubation the steroids were

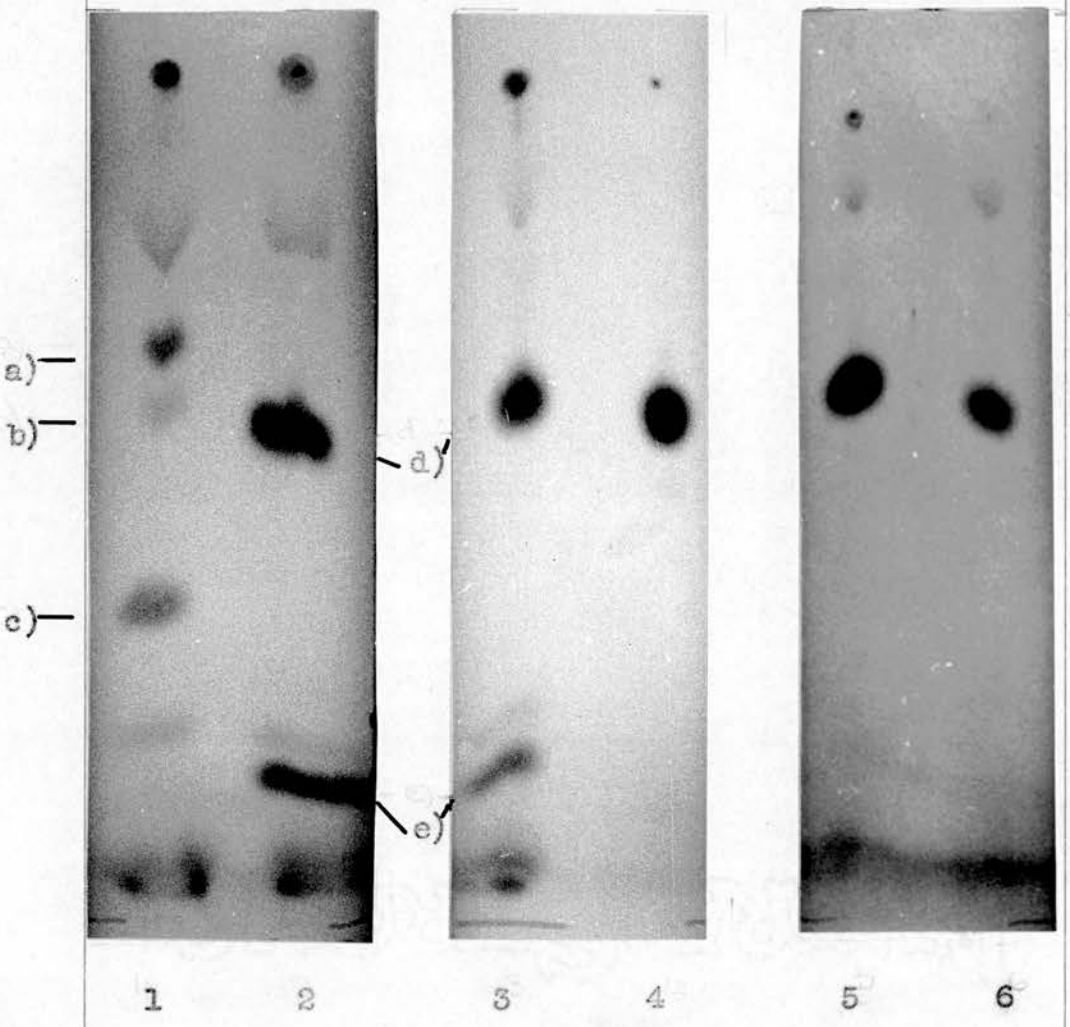


Fig. 49

Photographs of thin layer plates obtained from incubations with supernatant enzyme. Plates sprayed with phosphomolybdic acid.

1. Cholest-4-en-3-on-7α-ol as substrate
2. Coprostan-3-one-7α-ol as substrate
3. As 2
4. Standard 3α,7α-dihydroxycoprostane
5. Product isolated from an incubation of enzyme with coprostan-3-one-7α-ol, mixed with standard 3α,7α-dihydroxycoprostane
6. Product obtained from coprostan-3-one-7α-ol

Substances appearing on plates:

a) cholest-4-en-3α,7α-diol
b) 3α,7α-dihydroxycoprostane
c) cholest-4-en-3-one-7α-ol
d) incubation product of coprostan-3-one-7α-ol
e) coprostan-3-one-7α-ol

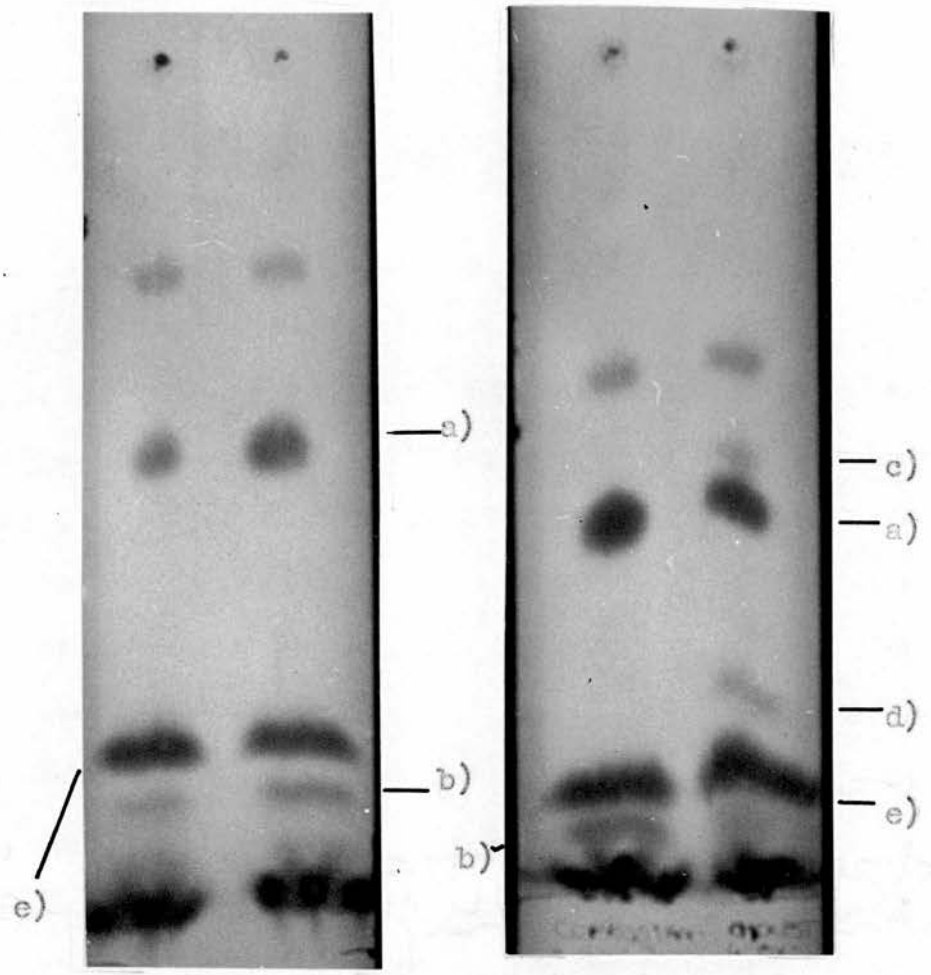


Fig. 49 (a)

Photographs of thin layer plates obtained from incubations of varying amounts of coprostan-3-one-7α-ol with supernatant enzyme and NADPH. Also included is a plate obtained from an incubation of cholest-4-en-3-one-7α-ol with the enzyme

1. 16 µg. coprostan-3-one-7α-ol as substrate
2. 24 µg. coprostan-3-one-7α-ol
3. 40 µg. coprostan-3-one-7α-ol
4. 42.5 µg. cholest-4-en-3-one-7α-ol

Substances appearing on plates:-

a) 3α,7α dihydroxycoprostane
b) coprostan-3-one-7α-ol
c) cholest-4-en-3α,7α-diol
d) cholest-4-en-3-one-7α-ol
e) cholesterol

extracted and the extracts run on thin layer plates which were then sprayed to show up the products. From the photograph (Fig. 49) it can be seen that coprostan-3-one-7α-ol, in the presence of enzyme and NADPH is very readily converted indeed into a substance with the same mobility as 3α,7α-dihydroxycoprostane. Indeed, by eye, it would appear that more than half of the added coprostan-3-one-7α-ol had been converted into dihydroxycoprostane. A control incubation stopped at zero time gave no such substance when extracted and run on thin layer chromatography. Cholest-4-en-3α,7α-diol was seen to give rise to a trace amount of dihydroxycoprostane.

Four incubations were set up, one of which contained 45 μg. of cholest-4-en-3-one-7α-ol and the other three containing varying amounts of coprostan-3-one-7α-ol (16, 24 and 40 μg). The incubations were otherwise identical and were treated in exactly the same manner. The lipid extracts were run on thin layer plates which were then sprayed to develop the products. The plates were photographed (Fig.49 a) and it can be seen that as the amount of coprostan-3-one-7α-ol as substrate increases, the amount of dihydroxycoprostane

formed increased. It can also be seen that 40 µg. coprostan-3-one-7α-ol gives rise to more dihydroxycoprostane than does 45 µg. cholest-4-en-3-one-7α-ol.

(b) Identification of the product as 3α,7α-dihydroxycoprostane

On spraying the thin layer plate obtained from an incubation of supernatant enzyme, NADPH and coprostan-3-one-7α-ol the only product seen had a mobility identical with that of authentic 3α,7α-dihydroxycoprostane in the benzene:ethyl acetate: acetone system, i.e., 0.43 (see Fig. 49). The product gave a yellow colour when sprayed with phosphotungstic acid as does 3α,7α-dihydroxy-coprostane. The product was eluted from an unsprayed plate, and the eluate taken to dryness. Concentrated sulphuric acid was added to the residue and the absorption spectrum of the yellow solution was plotted after one hour. The spectrum showed a peak at 312 mµ. similar to that given by 3α,7α-dihydroxycoprostane (Fig. 50)

The product of the reaction was eluted from an unsprayed plate, as above, and half of it was re-run on a similar plate mixed with authentic dihydroxycoprostane. The other half was also run

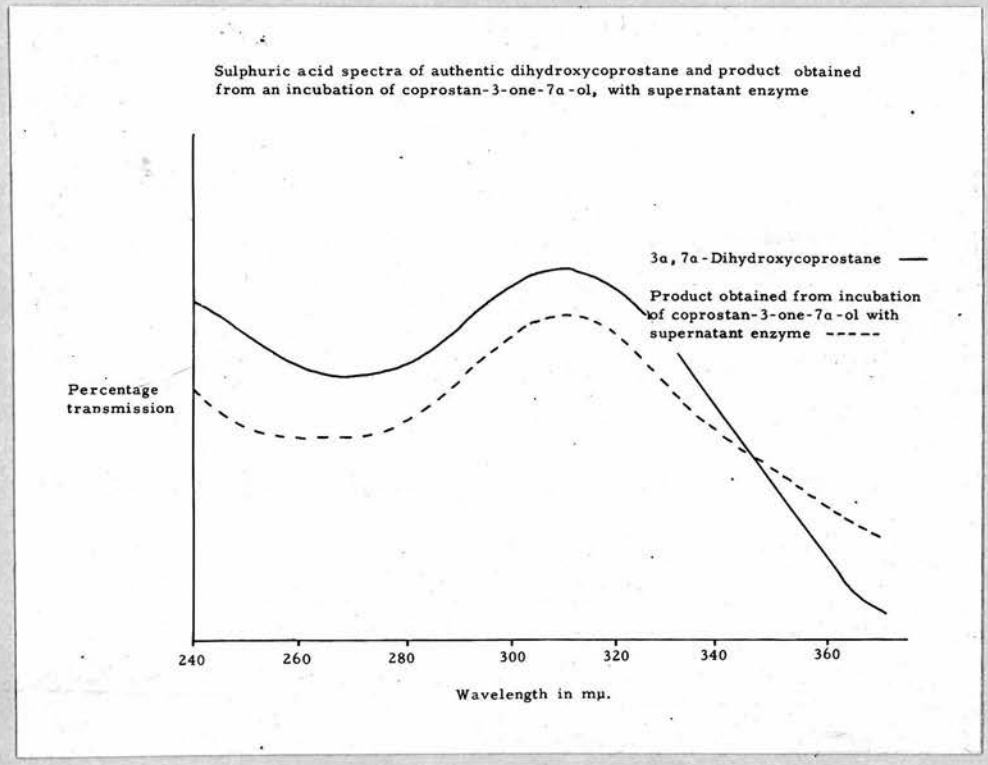

Sulphuric acid spectra of authentic dihydroxycoprostane and product obtained from an incubation of coprostan-3-one-7α-ol, with supernatant enzyme
3α, 7α-Dihydroxycoprostane
Product obtained from incubation of coprostan-3-one-7α-ol with supernatant enzyme
Percentage transmission
240
260
280
300
320
340
360
Wavelength in mμ.

Fig. 50

on the plate but not mixed with the pure 3α,7α-dihydroxycoprostane. In the benzene:ethyl acetate:acetone system the incubation product did not separate from the genuine compound. (Fig. 49).

This substantive evidence shows that when coprostan-3-one-7α-ol is incubated with this partially purified supernatant enzyme it is very readily converted into 3α,7α-dihydroxycoprostane.

(c) Co-factor requirements

(i) Incubations were set up to compare the effect of adding (a) NADPH-generating system, (b) NADH (equivalent to the amount of NADPH formed), and (c) no co-enzyme, on the formation of dihydroxycoprostane from coprostan-3-one-7α-ol.

Thin layer plates were run on the lipid extracts from such incubations and were sprayed firstly with phosphotungstic acid and then with phosphomolybdic acid. It was seen that dihydroxycoprostane was formed when either NADPH-generating system or NADH were added. No product could be detected when no co-enzyme was present.

Thus, the enzyme system which converts coprostan-3-one-7α-ol to dihydroxycoprostane is not specific for NADPH but functions equally well when NADH is added as co-factor.

(ii) An attempt was made to measure the amount of product formed when coprostan-3-one-7α-ol was incubated with varying amounts of NADPH from zero to 0.4 μ moles, prepared as described on p. 233. The product formed in each case was eluted from the unsprayed thin layer plate on which it was run, and to the residue, concentrated sulphuric acid was added. After one hour the solutions were read at 312 mμ and the number of μg. of dihydroxycoprostane formed was calculated from a calibration curve constructed by reading known amounts of dihydroxycoprostane in sulphuric acid at 312 mμ. However, this reaction is not a very sensitive one, and is not very accurate for quantities of dihydroxycoprostane less than about 50 μg. It was therefore impossible to draw any graph from the results. However, it was seen that there was no peak at 312 mμ. when 0 μ moles of NADPH were present, whereas at 0.25 μ moles NADPH about 25 μg. of dihydroxycoprostane was formed.

(d) Conclusion

It has been found in the above experiments that coprostan-3-one-7α-ol in the presence of either NADPH or NADH and enzyme is converted very

readily into 3α,7α-dihydroxycoprostane. This evidence, then, would indicate that two routes do exist for the formation of dihydroxycoprostane by reduction of cholest-4-en-3-one-7α-ol (see Fig. 39). As coprostan-3-one-7α-ol is so readily reduced to dihydroxycoprostane it presumably does not accumulate when cholest-4-en-3-one-7α-ol is incubated with the enzyme, and can therefore never be isolated from an incubation mixture.

On the other hand, as cholest-4-en-3α,7α-diol is not readily converted to dihydroxycoprostane, it does accumulate and can be isolated from the incubation mixture.

When cholest-4-en-3-one-7α-ol is incubated with enzyme and no co-factor, or with NADH, it has been shown that no products accumulate. It has also been shown that the reduction of coprostan-3-one-7α-ol requires either NADH or NADPH. This evidence suggest that the reactions reducing (a) cholest-4-en-3-on-7α-ol to cholest-4-en-3α,7α-diol, and (b) cholest-4-en-3-one-7α-ol to coprostan-3-one-7α-ol are both NADPH dependent.

The evidence obtained from the inhibitor studies indicates that cholest-4-en-3-one must inhibit, or be a substrate for the enzyme system which converts cholest-4-en-3-one-7αol to coprostan-3-one-7α-ol.

SUMMARY OF SECTION V

The metabolism of cholest-4-en-3-one-7α-ol was studied by incubating this substance, labelled or unlabelled, with the various cell fractions of rat liver. The methods employed lipid extraction, crude chromatography columns and the identification and estimation of metabolites using thin layer chromatography.

The results showed:-

(A) No metabolism of cholest-4-en-3-one-7α-ol occurred in the microsomal fraction.

(B) In mitochondria the more polar u.v. absorbing product was obtained. This hydroxylation reaction was studied under various conditions and it was found that:

a) the reaction was not stimulated by either NADPH or NADH;

b) SF (boiled supernatant) slightly stimulated the reaction;

c) Oxygen, as the gas phase, also slightly stimulatedthe formation of the product;

d) disruption of the mitochondria either sonically or osmotically did not destroy the enzymic activity, which was found to lie in the debris sedimented after disruption

e) an acetone powder of mitochondria was inactive

f) addition of the hydroperoxide of linoleate to mitochondria had no effect on the hydroxylation of cholest-4-en-3-one-7α-ol except under anaerobic conditions when the hydroxperoxide inhibited the hydroxylation reaction

(C) If either active supernatant fraction or SF were added to microsomes, the polar u.v. spot was produced from cholest-4-en-3-one-7α-ol . Thus a thermostable co-factor found in the supernatant fraction gives microsomes the power to hydroxylate. The co-factor was inactivated by treatment with alkali at 100°.

(D) The supernatant fraction was partially purified by an ammonium sulphate precipitation according to Tomkins.

1. (i) The substrate was found to be reduced to both cholest-4-en-3α,7α-diol and 3α,7α-dihydroxycoprostane

(ii) The production of both these substances was found to depend on -

a) enzyme concentration
b) substrate concentration
c) NADPH concentration

(iii) The production of dihydroxycoprostane was partially inhibited by the presence of cholest-4-en-3-one whereas the production of cholest-4-en-3α,7α-diol was not

(iv) Cholest-4-en-3α,7α-diol was found to be not readily converted into dihydroxycoprostane

(v) The enzyme system was not a specific one, as many Δ_4-3-ketones especially C_{21} steroids were readily reduced by it.

2. This partially purified supernatant fraction was able to convert coprostan-3-one-7α-ol very readily to dihydroxy-coprostane with either NADPH or NADH as co-factor, thus suggesting two possible routes for the formation of dihydroxy-coprostane by reduction of cholest-4-en-3-one-7α-ol (Fig. 39).

SECTION VI

METABOLISM OF 3α,7α-DIHYDROXYCOPROSTANE IN RAT LIVER CELL FRACTIONS

METABOLISM OF 3α,7α-DIHYDROXYCOPROSTANE IN RAT LIVER CELL FRACTIONS

It has been shown in this work, that cholest-4-en-3-one-7α-ol can be converted efficiently in the supernatant fraction of rat liver to 3α,7α-dihydroxycoprostane, which has been shown by Bergstrom and Lindstedt (1956) to be converted in the bile-fistula rat to both chenodeoxycholic acid and cholic acid. It would therefore be of interest to discover whether dihydroxycoprostane can be hydroxylated at position 12 to give 3α,7α,12α-trihydroxycoprostane. The mechanism of 12-hydroxylation may be even more complicated than other hydroxylations and the reaction may not be easily reproduced in vitro, as there has not been any evidence produced by workers in this field for the formation of 12-hydroxylated metabolites. Some very preliminary experiments were carried out using dihydroxycoprostane as a substrate and incubating it with the different cell fractions of rat liver.

(a) 3α,7α-dihydroxycoprostane was incubated with the three cell fractions of rat liver, mitochondria, microsomes and supernatant. Buffer,

pH 7.4, magnesium sulphate and nicotinamide were the only additions to the incubations. The incubation mixtures were extracted and columned as in the described method and the concentrated ether:alcohol cuts from the columns were run on fluorescent thin layer plates. The plates were examined under u.v. light and then sprayed with phosphotungstic acid. No u.v. absorbing products were seen to be formed in the incubations of any cell fraction. On spraying the plates, however, a more polar substance than dihydroxycoprostane was seen to be formed in the mitochondrial incubation only. Trihydroxycoprostane run as a standard in the same solvent system had a mobility similar to this substance. The substance gave a yellow colour with phosphotungstic acid, as does dihydroxycoprostane itself, and also trihydroxycoprostane.

(b) Two incubations were set up, containing dihydroxycoprostane as substrate. One of these contained boiled mitochondria and the other contained active mitochondria. The incubations were treated as usual and the section of the thin layer

plate obtained from the incubation with active tissue which should contain the polar substance seen to be formed in the first experiment, was scraped off, eluted with ether:alcohol and re-run on a thin layer plate alongside standard trihydroxycoprostane. The rest of the first plate was sprayed with phosphotungstic acid and it was seen that no polar substance with the same mobility as the substance formed in the active incubation, was produced in the boiled tissue incubation. On spraying the second plate, which was also run in the benzene:ethyl acetone:acetone system, the polar substance produced in the active incubation was found to be slightly less polar than 3α,7α,-12α-trihydroxycoprostane. This would indicate that the substance is probably 26-hydroxylated derivative of 3α,7α-dihydroxycoprostane as the addition of a 12-hydroxyl group would make a substance more polar than the addition of a 26-hydroxyl group.

Active mitochondria can therefore convert 3α,7α-dihydroxycoprostane to a more polar substance, probably the 26-hydroxylated derivative.

SECTION VII

DISCUSSION

DISCUSSION

A brief summary of the progress achieved in this study is given in the following paragraph:-

a) A method has been developed whereby small amounts of the possible intermediates in cholesterol breakdown, can be incubated with rat liver cell fractions.

b) A study of the initial hydroxylation of cholesterol has been undertaken.

c) The study of the metabolism of 7α-hydroxycholesterol in the different cell fractions of rat liver and combinations of cell fractions has led to some interesting discoveries.

d) It has been established that the possible intermediate in cholesterol breakdown, cholest-4-en-3-one-7α-ol, is metabolised efficiently in rat liver to 3α,7α-dihydroxycoprostane, a knownintermediate. Cholest-4-en-3-one-7α-ol is also converted to a more polar compound, possibly the 26-hydroxylated derivative.

d) Preliminary experiments show that 3α,7α-dihydroxycoprostane is converted to a more polar compound also.

The method of extraction and purification of metabolites that has been developed in this work has the advantage that many incubations can be carried out on the cell fractions from any one rat liver. This means that not only can small amounts of substrate be used, but also each rat acts as its own control. This is important as there is no doubt that there is variation from one rat liver to another. This may depend on the age of the animal but could also depend on the levels of co-enzymes occurring in each preparation. Several examples have been cited in the text, especially where mitochondrial preparations are concerned when it has been suggested that certain results are unusual because of a low or high level of NAD in the preparation (see pages 141 and 152). A further advantage of being able to carry out several incubations on the cell fractions from one rat liver, is that an effect, such as substrate concentration variation or co-enzyme concentration variation, can be studied, although the cell fractions have not been further purified.

The development of thin layer chromatography and very especially the development in this study of the fluorescent thin layer plates has proved to be of importance in this method. The method, however, does have the disadvantage that substances must be eluted from the plate before estimation and a column method of separation of metabolites giving a direct estimation of substances, could, perhaps, be more convenient. However, it is likely that no simple and quick column method could give such complete separation of compounds as thin layer chromatography, and probably only on reverse phase columns such as used by Danielsson (1958) could such separation be achieved. The reverse phase system of columning is slow and also susceptible to temperature change. Danielsson used mg. amounts of substances and it might be difficult to adapt this reverse phase system to a micro-scale. The reverse-phase system then is not suited to the rapid separation of the many metabolites obtained from incubations some of which are only present in amounts of 5 µg. or less. An advantage of thin layer chromatography over such a column system is that it gives

not only a method of separation but also a direct way of identifying metabolites at one and the same time.

With the method, that has been developed, no attempt has been made to detect the formation of acidic metabolites such as chenodeoxycholic acid and cholic acid or the corresponding coprostanic acids. However, with modifications in the extraction procedure, the columning and the solvent systems used for thin layer chromatography the method could easily be adapted to study this aspect.

Thus, it seems that this method allows the study of the metabolism of possible intermediates in cholesterol breakdown in rat liver incubations with the minimum time taken for results to be obtained. Metabolites can be identified and estimated even when only 2% of the substrate has been converted to a product.

Some preliminary experiments have been carried out to try to discover how and where the initial hydroxylation of cholesterol takes place.

Using cholesterol as a substrate presents the problem of autoxidation, as has been pointed out, and the experiment performed incubating

labelled cholesterol with the different cell fractions of rat liver failed to give much useful information, partly because of this. In this experiment the results obtained by Danielsson (1960) were not confirmed. This is probably because he used mouse mitochondria and mitochondria plus SF, with additions such as NAD, AMP, etc. and also used SF only as a control to indicate the degree of autoxidation. The cholesterol used in the experiment performed in Section III, was probably not sufficiently radioactive, which meant that small changes were very difficult to identify. Thus, if small amounts of metabolites had been formed, their presence might have been overlooked. Thus, it seems that about the only information to be gained from the experiment is that autoxidation is particularly marked in the microsomal fraction. An interesting point is that in the active mitochondrial incubation, the radioactivity recovered from the 7α-hydroxycholesterol segment was actually less than that obtained from the corresponding segment of the boiled mitochondria incubation. It might be possible, then, that as the 7α-hydroxycholesterol was formed from cholesterol, it was converted to a more polar

product such as cholest-5-en-3β,7α,26-triol. This hydroxylation reaction has been found to occur in mitochondria (see Section IV, p. 139) with no additions. The radioactivity recovered in the origin segment of the active mitochondrial incubation, where the triol would run was higher than that obtained from the boiled incubation, which would agree with this theory.

In an attempt to discover the mechanism of the initial hydroxylation at the 7α-position, the 7α-hydroperoxide of cholesterol was used as a substrate. It is possible that in the formation of 7α-hydroxycholesterol, a hydroperoxide is formed as an intermediate which is then reduced by some agent such as NADPH. Chemically, it is readily reduced to 7α-hydroxycholesterol by lithium aluminium hydride. However, the study of the metabolism of this substance is made very difficult by its instability, even to incubation at 37° with buffer, when both 7α-hydroxycholesterol and 7-ketocholesterol are produced, the latter probably by dehydration. Adding the reducing co-enzyme NADPH to the buffer incubation gave no increase in the amount of 7α-hydroxycholesterol formed. Thus, NADPH cannot reduce the substrate in buffer solution only. On adding an acetone powder of mitochondria,

however, about double the amount of 7α-hydroxy-cholesterol was formed. Addition of NADPH to the acetone powder made no increase in the amount formed. It may be, then, that possibly enzymic activity does exist in mitochondria which will catalyse the reduction of the 7α-hydroperoxide but the reduction could just as easily be brought about by a reducing agent such as glutathione present in the acetone powder. On comparing the activities of boiled and active mitochondrial powder it was shown that in the active incubation more 7α-hydroxy cholesterol was produced than in the boiled incubation, indicating that some enzymic activity does exist. Any attempt to study the metabolism of the 7α-hydroperoxide in native tissue cell fractions will be made very difficult by the instability of the compound and adequate controls will have to be incluced in any series of incubations.

It would be of interest to discover whether labelled 7α-hydroperoxide would be converted to bile acids in the bile-fistua rat.

The study of the metabolism of cholesterol by rat macrophages maised some interesting points. This tissue preparation was found to be clean to

work with, no column procedure was required to purify the constituents of the lipid extracts and also autoxidation was at a minimum although after long periods of incubation, it became more noticeable. It was found in the experiments carried out, however, that in any series of incubations stopped at different time intervals, no increase in products could be seen with increase in time. In fact, it seemed that either the incubation was inactive in metabolising cholesterol or was very active. This is probably due to the condition of the cells. Sometimes for unknown reasons, the macrophages do not grow satisfactorily and tend to slough off the surface of the "baby bottle". Thus, variations in results are probably due to this.

When an incubation was active, however, it seemed to produce fairly readily, 7α-hydroxycholesterol and more polar substances from cholesterol or its esters. In one experiment bile acids have been identified as products. This result does not agree with the findings of Day, (1961) who showed that no oxidation of the side-chain of cholesterol was brought about by rabbit macrophages. However, he was using cholesterol

as a substrate and in the experiments conducted in this study a mixture of cholesterol and cholesterol esters as they occur in serum was employed. This would imply that cholesterol esters may be the starting compounds for the breakdown of cholesterol to bile acids. The possibility that the bile acids found in the incubation described in Section III p. 95 may have been present in the serum must not be overlooked however. Also Day used rabbit macrophages, whereas in this work rat macrophages were employed. It is interesting to note that a substance with chromatographic properties identical to 7α-hydroxycholesterol was present in minute quantity in the rat serum. This substance was found to be non-radioactive.

This type of experimental approach shows great promise in the study of cholesterol breakdown and it would be of interest to discover whether 7α-hydroxycholesterol and cholest-4-en-3-one-7α-ol and other possible breakdown intermediates of cholesterol can be metabolised by these cells. In the case of substrates, other than cholesterol, they would have to be added to the incubation in some medium other than serum, such

as methanol or albumin suspension. Check experiments would then have to be performed to find out if methanol had any adverse effect on the growth of the macrophages.

The study of the metabolism of 7α-hydroxycholesterol in the different cell fractions of rat liver and combinations of cell fractions has brought to light some interesting points.

As has been discussed in the Introduction, it certainly seems that rat liver and mouse liver can convert 7α-hydroxycholesterol to the less polar, oxidised substance, cholest-4-en-3-one-7α-ol, presumably being the intermediate formed during the epimerisation of the 3β-hydroxyl group and the saturation of the double bond. However, there seems to be some doubt as to where in the cell this conversion is carried out. Yamasaki et al. (1959) have found activity for producing an α,β-unsaturated ketone from 7α-hydroxycholesterol in what they call "the supernatant fraction" of rat liver whereas Danielsson (1961 b) finds the formation of cholest-4-en-3-one-7α-ol to take place in the mitochondria of mouse liver to which SF has been added. The results obtained in this work, however, do not agree with either of these findings.

In this work, then, two enzyme systems, both presumably containing 3β-hydroxy dehydrogenase and isomerase activities have been found, both of which can convert 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol. One of these enzymes is located in the mitochondria and is dependent on either NAD or NADP as electron acceptor. This may be the enzyme which gives the activity described by Danielsson (1961 b) in mouse mitochondria. The other enzyme found in this work, is considerably more efficient at converting 7α-hydroxycholesterol to the oxidised compound than is the mitochondrial enzyme. In fact, for the same weight of liver and the same amount of coenzyme, the microsomal enzyme is nearly four times as active as the mitochondrial enzyme. This microsomal enzyme may be the enzyme described by Yamasaki et al. (1959) as producing an α,β-unsaturated ketone from 7α-hydroxycholesterol, as Yamasaki prepared the "supernatant fraction" by spinning the homogenate free from nuclei and cell debris, at 80,000 x g for 30 min. It is doubtful if this procedure would completely sediment the microsomes so that Yamasaki's "supernatant fraction" might contain some microsomes. In this

work, only 2% of the substrate was ever found to be converted to cholest-4-en-3-one-7α-ol in the 100,000 x g supernatant, and this may be due to unsedimented microsomes.

It was thought that perhaps the two oxidative enzymes discovered in this work might be one and the same and that the mitochondrial activity was merely due to microsomal contamination. There are in fact several similarities between the two activities. Firstly, both are dependent on NAD as electron acceptor, which can be replaced in both mitochondrial and microsomal systems by NADP. Secondly, the effect of cyanide on these enzymes is very similar. In both cases, contrary to expectation, adding cyanide increased the amount of cholest-4-en-3-one-7α-ol formed from 7α-hydroxycholesterol. This is thought to be due to the cyanide, inhibiting other NAD-requiring reactions occurring in the two cell fractions, and thus making more NAD available for the oxidation of 7α-hydroxycholesterol.

However, even although the two oxidative enzymes are similar in these two ways, the following points would seem to disprove the suggestion that only one enzyme systems exists. Firstly,

on varying the substrate concentration added to incubations with mitochondria and with microsomes the same amount of co-enzyme being added to each series of incubations, it was found that the curves obtained on plotting substrate concentration against reaction velocity, (i.e. amount of product formed/hr.) were different in shape, (see pages 114 & 127). Thus, the K_m values for both enzymes were significantly different i.e. K_m for microsomal enzyme is 3.13×10^{-4}M and K_m value for mitochondrial enzyme is 3.8×10^{-5}M.

Secondly, the effect of succinate on the two enzymes is quite different. Adding succinate to the microsomes has no effect on the oxidative activity whereas on addition to mitochondria it has a profound effect. In mitochondria adding succinate gives rise to hardly any cholest-4-en-3-one-7α-ol and in some cases the formation of product is completely inhibited. This is thought to be due to the fact that succinate is reducing all the added NAD to NADH in mitochondria, under the influence of succinic oxidase (Chance, 1956). It may be that no such activity for reducing NAD to NADH with the aid of succinate occurs in microsomes but respiratory activity is certainly present in this cell fraction (Ernster, 1958; Penn and Mackler,

1958).

Thirdly, the effect of SF on the two enzymes is different. In mitochondria, as will be discussed in detail, it has the effect of reducing the amount of product formed by the enzyme by more than half, two more polar products being simultaneously formed. In microsomes, the addition of SF gives rise to a slight reduction of the cholest-4-en-3-one-7α-ol formed, and the formation of one more polar product.

Thus two enzymes do exist which will oxidise 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol and probably the most important one is to be found in the microsomal fraction of rat liver.

In this study it has been found that SF plays a part in certain hydroxylation reactions occurring in the breakdown of cholesterol. The role of SF was hitherto, unknown although it was found to be necessary for the oxidation of the side-chain of cholesterol.

The effect that SF seems to have on the binding of substrates to tissue is interesting but may not be of any significance. It was shown that 100% recovery of labelled 7α-hydroxycholesterol could be obtained from an incubation

with boiled mitochondria and SF, whereas only about 85% could be achieved if active mitochondria and SF were involved (i.e. 85% of the added counts, some of which were associated with the products of the reaction). A similar state of affairs was found to exist when labelled cholest-4-en-3-one-7α-ol was used as a substrate. This effect may only be coincidental and not important in the metabolism of either of these substances.

SF was found, however, to have a profound effect on the metabolism of 7α-hydroxycholesterol in the mitochondrial fraction of rat liver. If mitochondria are incubated with 7α-hydroxycholesterol and NAD, about 6% of the substrate is converted into the oxidation product, cholest-4-en-3-one-7α-ol. If, however, SF is added to such an incubation mixture, the amount of cholest-4-en-3-one-7α-ol formed is reduced to about 2% and at least two other more polar metabolites are produced. The percentages of these polar products which are thought to be hydroxylated derivatives add up to about 4%, the amount by which the oxidation product is reduced. Therefore it appears that SF "directs" the metabolism of 7α-hydroxycholesterol away from an oxidation reaction and towards hydroxylation reactions.

In the absence of NAD or SF, mitochondria are capable of converting 7α-hydroxycholesterol to one of the more polar products, cholest-5-en-3β,7α,26-triol. Therefore if this substance is produced on incubation of 7α-hydroxycholesterol with mitochondria, and also with mitochondria, SF and NAD, SF must act by cancelling out the effect of the NAD. An experiment in which mitochondria were incubated with varying concentrations of NAD supported this idea, because it was found that as the NAD concentration increased, the amount of cholest-4-en-3-one-7α-ol formed increased and the amount of triol formed decreased. An incubation containing a low concentration of NAD was therefore equivalent to an incubation containing a higher concentration of NAD and SF. If this is the case, then, that SF acts partially be eliminating the effect of NAD, the question to be answered is, how does it have this effect? It seems that SF must either inactivate the NAD in some way, or cause its removal by metabolising it. The latter explanation seems the most likely.

Considerable interest has been aroused recently by the possibility that dicarboxylic acids, in particular, succinate, may control the ratio of NAD:NADH in the mitochondria (Chance, 1956; Snoswell, 1962) by reducing NAD to NADH. When succinate was incubated with mitochondria, NAD and 7α-hydroxycholesterol in place of SF, it was found to have the same activity as SF, giving a lower conversion of substrate to cholest-4-en-3-one-7α-ol and giving rise to the two hydroxylated metabolites. In this case, one can be confident that succinate is acting in this way by reducing the added NAD to NADH and thus limiting the formation of the oxidation product of the substrate. Other dicarboxylic acids such as fumarate, oxaloacetate, α-ketoglutarate, which would also stimulate the removal of NAD, were found to mimic the effect of SF. Dicarboxylic acids, which would not, however, cause the removal of NAD, such as maleate, malonate and dihydroxyfumarate were found to have no SF-like activity when incubated with mitochondria and NAD and actually gave use to slightly more of the oxidation product of 7α-hydroxycholesterol than mitochondria with only NAD added. This might be due to these

substances inhibiting the normal NAD-requiring reactions occurring in mitochondria, and thus making more NAD available for the oxidation of the substrate. Citrate, the tricarboxylic acid was found to be as active as succinate in its effect on the metabolism of 7α-hydroxycholesterol in mitochondria. Chance (1956) found that succinate reduces NAD to NADH more efficiently than the conventional NAD-linked intermediates in the citric acid cycle. In this case, then, it is probable that when succinate is added to an incubation it is acting merely as a citric acid intermediate stimulating the removal of NAD by stimulating the citric acid cycle, and is not taking part in the reaction described by Chance where succinate directly reduces NAD to NADH.

The theory that SF has part of its effect by reducing the added NAD is thus strengthened by these findings and also by the fact that incubating NADH with mitochondria and substrate gives the same result as incubating mitochondria with SF and NAD. It is not unlikely, then, that SF contains a substance with similar properties to succinate or fumarate. It seems however, that this property of SF of removing added NAD is only

one part of its activity. If mitochondria are incubated with 7α-hydroxycholesterol and various amounts of SF, then the amount of triol formed increases as SF increases. Thus, SF may also actually stimulate the process of hydroxylation, as well as depleting NAD. This "stimulation" of hydroxylation reactions by SF is also seen to occur when cholest-4-en-3-one-7α-ol is incubated with mitochondria. This "stimulation" might be explained if SF was merely reducing any endogenous NAD to NADH, causing the following reactions to take place:-

$$NAD \xrightarrow{SF} NADH$$

$$NADH + NADP \longrightarrow NADPH + NAD$$

NADPH, normally required for hydroxylation reactions, would then stimulate the hydroxylation reaction. If this was the case, one would expect succinate to have the same effect. It was found however, that incubating 7α-hydroxycholesterol with mitochondria and varying amounts of succinate gave no increase in the amount of triol formed. Also adding NADPH to incubations of mitochondria with cholest-4-en-3-one-7α-ol gave no increase in the amount of the hydroxylated derivative formed, whereas SF did give increased formation. It

would seem, then, that SF does not stimulate hydroxylation by this means and this also shows that SF has two types of activity; firstly, it has the power to remove NAD, and this activity may be quite coincidental, and secondly, it has the power to stimulate hydroxylation reactions.

In the microsomal fraction of rat liver also it was found that SF plays a part in hydroxylation reactions. Microsomes, with no additions, unlike mitochondria, are incapable of hydroxylating either 7α-hydroxycholesterol or cholest-4-en-3-one-7α-ol. If an incubation is carried out with microsomes, NAD and 7α-hydroxycholesterol the oxidation product, cholest-4-en-3-one-7α-ol is formed, very efficiently. On addition of SF (or active supernatant) to such an incubation the more polar u.v. absorbing compound, the hydroxylated derivative of cholest-4-en-3-one-7α-ol is formed, together with slightly less of the latter substance. This means that SF is causing the microsomes to hydroxylate this substance. This fact is confirmed by adding SF to an incubation involving microsomes and cholest-4-en-3-one-7α-ol as substrate, and showing that hydroxylation occurs. This does not happen, as has been pointed out, in

MICROSOMAL HYDROXYLATION REACTIONS

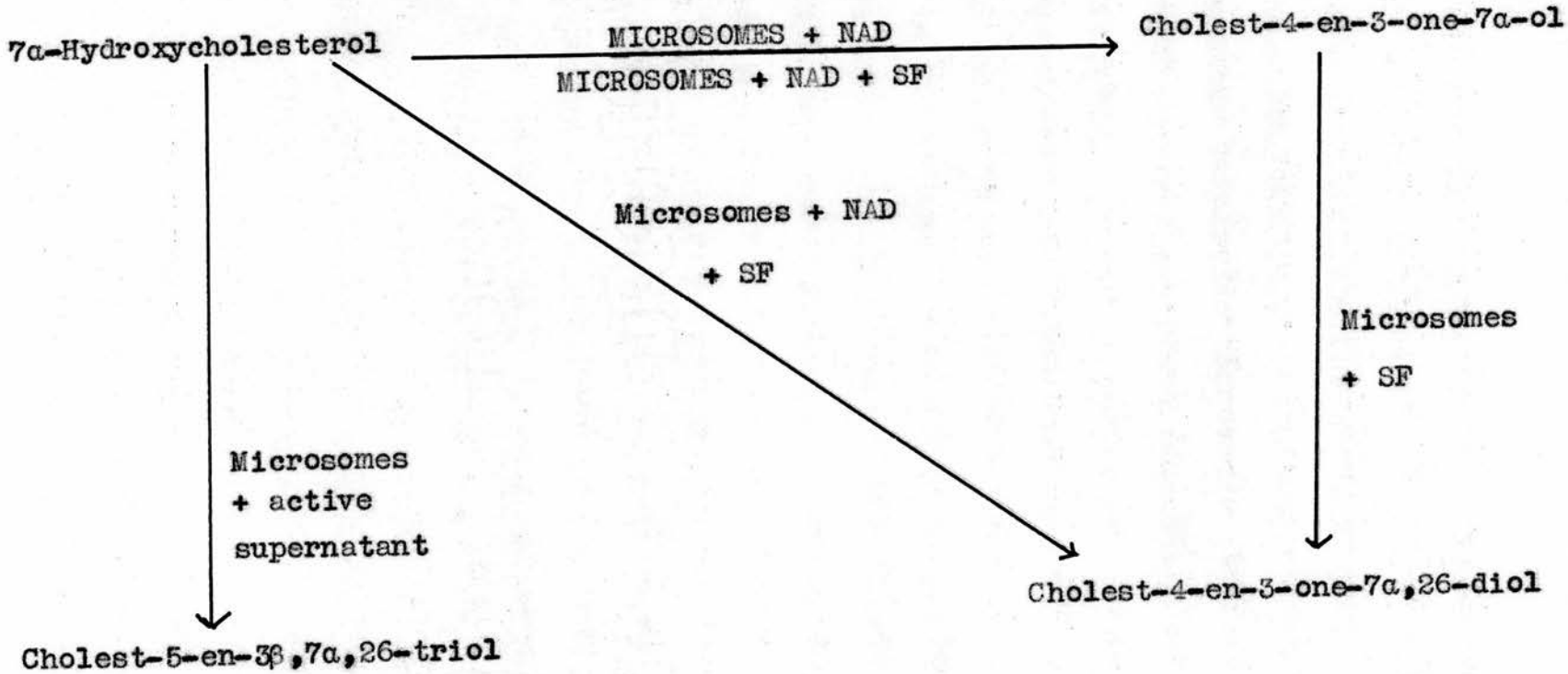


Fig. 52 (a)

incubations with microsomes and substrate alone. These reactions are summarised in Fig. 52 (a).

This situation, then, is rather similar to that occurring in the mitochondrial fraction, in that SF, apparently stimulates hydroxylation. In the microsomal fraction, however, SF cannot presumably be acting by removing added NAD. In incubations of microsomes NAD, 7α-hydroxybholesterol and SF, the hydroxylated substance formed is a derivative of the oxidation product, and not, as occurs in mitochondria a derivative of 7α-hydroxycholesterol itself. Therefore, the oxidation product must be formed first, and then hydroxylated and no depletion of NAD can take place.

This latter theory is strengthened by the fact that adding succinate to microsomes, NAD and 7α-hydroxycholesterol in place of SF, has no effect at all. No hydroxylated derivative is formed and the amount of cholest-4-en-3-one-7α-ol formed is not significantly reduced. It may be that no mechanism, such as that occuring in mitochondria for reducing NAD with succinate or SF, exists in microsomes. Succinate cannot, then, replace SF in this microsomal hydroxylation mechanism. Also it has been found that adding NADH,

to microsomes with cholest-4-en-3-one-7α-ol as substrate does not have the same effect as adding SF. In other words, NADH cannot replace the activity of SF in giving microsomes the power to hydroxylate. Again this is a different situation from that existing in mitochondria where NADH can replace NAD + SF.

The evidence summarised here, emphasises the fact that SF has two types of activity. In the mitochondrial fraction, firstly, SF can deplete added NAD probably by reduction to NADH, and secondly the SF stimulates hydroxylation. In microsomes the latter type of activity only is found to exist. SF is found, in this case, to be required before hydroxylation occurs, microsomes with no additions being unable to carry out hydroxylation. The activity of SF to reduce NAD in mitochondria may be quite unimportant but as this effect complicates the situation occurring in mitochondria, the activity of SF to promote hydroxylation might be better studied in microsomes.

A very preliminary experiment designed to obtain some knowledge as to nature of SF, showed that the activity of SF in mitochondrial incubations with 7α-hydroxycholesterol and NAD, was

resistant to treatment with boiling acid, but on treatment with boiling alkali, its activity was completely lost. This means that SF is probably not succinate or any other citric acid intermediate, which have been shown to have the same effect as SF in depleting NAD, but not in stimulating hydroxylation, as these substances should withstand such treatment. SF might possibly be a phosphate ester, such as ATP or some similar substance, which would be hydrolysed by alkali under these conditions but not by acid. A similar situation was also found to occur when acid and alkali-treated SF were incubated with microsomes and cholest-4-en-3-one-7α-ol, "acid-SF" retaining its ability to promote hydroxylation, and "alkali-SF" being inactive. In this case, no complications due to NAD depletion are involved.

The fact that inactive (alkali-treated) SF is bright yellow in colour, might be a clue to the nature of SF. The co-factor required in the hydroxylation of phenylalanine to tyrosine has been found by Kaufman (1962) to contain a pteridine moiety, and many pteridine derivatives are yellow in colour. Kaufman's co-factor was rendered inactive by treatment with alkali at 100°, with

the formation of a fluorescent compound, but was not, however, stable to freezing. Thus it is unlikely that SF is the same substance as Kaufman's co-factor.

It has been found, in this work the hydroxylation of cholest-4-en-3-one-7α-ol still occurs in mitochondria under anaerobic conditions. Initially, this was thought to be due to incomplete anaerobiosis, but another explanation would be that a bound form of oxygen, such as a peroxide exists in native mitochondria which could play a part in the hydroxylation of the steroid. Under anaerobic conditions linoleate hydroperoxide actually inhibits hydroxylation, but there is a possibility that SF itself could be a similar form of activated oxygen and that, because of this it has the ability to stimulate hydroxylation reactions. However, any attempt to replace SF by the hydroperoxide of linoleate has been unsuccessful. An experiment set up comparing incubations of mitochondria and 7α-hydroxycholesterol and mitochondria, SF and 7α-hydroxycholesterol both under anaerobic conditions, might give results which would clarify the situation. If, under anaerobic conditions, hydroxylation of 7α-hydroxy-

cholesterol could not occur, then the addition of SF to such a situation might cause hydroxylation. to occur. This would indicate that SF could, in fact, provide the oxygen required in the reaction.

It is possible that the same mechanism for the hydroxylation of all steroids, exists. NADPH has been shown to be absolutely necessary in many instances where purer enzyme preparations have been studied and it is to be expected that this requirement will in due course be noted for all hydroxylations. The 11-β-hydroxylase enzyme has had the most extensive study of all the steroid hydroxylases and Tomkins et al. (1958) found that the "co-hydroxylase"factor required in the reaction could not be replaced by any of the following substances; ascorbic acid, glutathione, nicotinamide fumarate, citrate, NADPH, hydrogen peroxide, folic acid or Kaufman's phenylalanine-hydroxylating co-factor. It would be of interest, then to discover whether any of these substances could replace SF in its activity to stimulate hydroxylation of cholesterol derivatives.

Metal ions have also been shown in many cases to be necessary for steroid hydroxylations and their participation is assumed to be in the

activation of oxygen. The addition of the chelating agent EDTA to the system in microsomes which with SF, hydroxylates cholest-4-en-3-one-7α-ol might show an inhibition of the reaction which would indicate that SF was contributing a metal ion.

However, in order to study the nature of SF with any success, a hydroxylating system must be found with a fast assay procedure. As has been pointed out the amount of triol or polar u.v. absorbing material obtained either with mitochondria or microsomes is very small indeed and also the enzyme system in mitochondria is not very stable. This is seen by its inactivation on treatment with acetone and therefore an attempt to purify the enzyme system to give a faster assay procedure, might inactivate the system entirely.

If the amount of hydroxylated derivative formed could be increased in some way, probably the best system in which to study the effect of SF on hydroxylation would be the microsomal system, where the polar u.v. absorbing material is produced from cholest-4-en-3-one-7α-ol on addition of SF. The amount of hydroxylated derivative might be increased by using a much longer

incubation time or by the addition of other co-factors such as ATP. The fact that both product and substrate in this reaction are both u.v. absorbing means that a method dependent on the fluorescent thin layer plate technique is very suitable. If cholest-4:6-dien-3-one could be used as a substrate, then both substrate and the hydroxylated product would have an absorption peak in the u.v. range at 282 mμ. with an extinction co-efficient of about 28,000. This means that small amounts of product would be more easily identified and estimated than the corresponding amount of "polar u.v. absorbing material" with an extinction co-efficient of 16,000.

The SF used in all these experiments has been prepared exactly as described in the original paper by Anfinsen and Horning (1953). Whether, in fact, the stimulatory effect of SF on hydroxylation reactions, found in this work, is the same effect that has been studied on the oxidation of cholesterol to CO_2 is not known. Hydroxylation of the side chain presumably occurs before the side-chain is split off and so the effect of SF, seen in stimulating side-chain removal may well be a reflection of its effect on stimulating hydroxy-

lation reactions. It would be of great interest to discover whether SF only stimulates hydroxylation of the side-chain at position 26 or whether it also stimulates 12α- or 7α-hydroxylation. Two pathways must exist for the separate formation of cholic acid and chenodeoxycholic acid from cholesterol. This is so because substances such as 26-hydroxycholesterol or cholest-5-en-3β,7α,26-triol give rise only to chenodeoxycholic acid in the bile-fistula rat, indicating that 12α-hydroxylation cannot take place after modification of the side-chain. Chenodeoxycholic acid itself cannot be transformed into cholic acid. This means that if SF stimulates hydroxylation at position 26, as has been shown here, it has a key role in directing the metabolism of cholesterol so that either chenodeoxycholic acid or cholic acid is formed.

The formation of the 26-hydroxylated derivatives of the two compounds, 7α-hydroxycholesterol and cholest-4-en-3-one-7α-ol appears to be a complicated procedure. In this work, the most easily studied and most efficient reactions have been those of oxidation and reduction. Very small amounts of these hydroxylated derivatives have ever been produced and the enzyme system is

sensitive to acetone treatment. However, if 26-hydroxylation of these compounds is a necessary step in the pathway to chenodeoxycholic acid, then one might expect these enzymes to be less active as usually 20% of the bile acids produced from cholesterol is chenodeoxycholic acid whereas 80% is cholic acid.

In mitochondria, to which NAD is not added, 7α-hydroxycholesterol is converted to cholest-5-en-3β,7α,26-triol and this is the only product formed. Adding SF to mitochondria stimulates the formation of this substance. In microsomes, however, no triol is formed from 7α-hydroxy-cholesterol unless active supernatant fraction is added. SF cannot replace the active supernatant in this respect and it would therefore seem that some thermolabile factor, such as an enzyme existing in the supernatant fraction is required by the microsomes before hydroxylation can occur. Supernatant alone cannot hydroxylate 7α-hydroxy-cholesterol either. Thus, two enzymes are probably required for hydroxylation.

The polar u.v. absorbing substance, thought to be cholest-4-en-3-one-7α,26-diol is formed when mitochondria are incubated with cholest-4-en-

3-one-7α-ol. This reaction has been studied under various conditions and it is interesting to discover that the enzyme (or enzymes) responsible for the reaction are located in the mitochondrial debris which might mean that hydroxylation occurs in the wall of the mitochondrion. It is not really surprising that the addition of NADPH does not stimulate the reaction in such a crude preparation, as 50% of the cell NADP is found in the mitochondria.

The polar u.v. absorbing substance is also seen to be produced under various conditions when 7α-hydroxycholesterol is used as substrate in mitochondria. This formation is discussed on p. 168 and the conclusion reached is that it must be formed by oxidation of the triol, rather than by hydroxylation of cholest-4-en-3-one-7α-ol, which is perhaps contrary to expectation considering that cholest-4-en-3-one-7α-ol can be converted to the u.v. absorbing substance by mitochondria. This theory was confirmed by incubating triol with mitochondria and NAD and finding that the polar u.v. absorbing substance was formed.

In the microsomal fraction it has been found that no conversion of cholest-4-en-3-one-7α-ol to

the polar u.v. absorbing substance occurs unless SF is added to the incubation. Using 7α-hydroxycholesterol as substrate, the only product formed on incubations with microsomes and NAD is cholest-4-en-3-one-7α-ol. Addition of active supernatant or SF, however, then gives rise to the polar u.v. absorbing substance, together with cholest-4-en-3-one-7α-ol. In this case, then, the polar u.v. absorbing substance must be formed by hydroxylation of cholest-4-en-3-one-7α-ol and not by oxidation of the triol as no triol is formed under these conditions.

It has been found that 7α-hydroxycholesterol can be esterified in the supernatant fraction of rat liver. Deykin and Goodman (1962 a) have found that only hydrolysis of cholesterol esters occurs in this fraction whereas esterification takes place in the microsomal or mitochondrial fraction on the addition of co-enzyme A and ATP, (Deykin and Goodman, 1962 b). Some esterification of 7α-hydroxycholesterol has also been found, in this work to occur in the mitochondrial fraction particularly on the addition of SF. Fredrickson, (1956) investigating the products formed from C^{14}-labelled cholesterol in the mouse mitochondria

system of Anfinsen and Horning (1953) which includes SF, found that 3% of the products formed could be attributed to cholesterol esters. Thus, there is an esterifying system present in mouse mitochondria and probably such a system exists in rat mitochondria also. In the studies in this work on the metabolism of C^{14}-labelled cholesterol however, no esterification was seen to take place in any of the rat liver cell fractions.

It is not know whether esterification of 7α-hydroxycholesterol is a significant reaction or whether 7α-hydroxycholesterol is merely acting as a substrate for the enzymes which normally esterify cholesterol. The latter may be the case in the mitochondrial system, but as hydrolysis of cholesterol esters normally occurs in the supernatant fraction the esterification of 7α-hydroxycholesterol noted here may be of some importance.

Mawer (1962) isolated 7α-hydroxycholesterol esters from various sources including rat serum, skin and liver and human serum from patients fed safflower seed oil. It is not knownwhether the 7α-hydroxycholesterol esters found by Mawer, are formed by hydroxylation of cholesterol at 7α-position, followed by esterification or by

esterification of the cholesterol initially and then hydroxylation. A few experiments carried out in this study incubating 7α-hydroxycholesterol laurate with the cell fractions of rat liver showed that hydrolysis of the ester to 7α-hydroxycholesterol occurred readily. This would seem to imply that if 7α-hydroxycholesterol esters are formed in vivo, that they are probably produced by hydroxylation of cholesterol esters.

However, if 7α-hydroxycholesterol esters are formed normally from cholesterol esters a possible explanation can be given for their production from 7α-hydroxycholesterol when it is incubated with the supernatant fraction of rat liver. It may be than an ester grouping is necessary to protect the 3β-hydroxyl group, while reactions at other positions in the steroid are occurring.

It has been established, in this study, that cholest-4-en-3-one-7α-ol to which 7α-hydroxycholesterol is readily converted in microsomes, is metabolised in rat liver to 3α,7α-dihydroxycoprostane. Both cholest-4-en-3-one-7α-ol and 3α,7α-dihydroxycoprostane have been shown to be converted to bile acids in the bile fistula rat (Danielsson, 1961 a; Bergstrom and Lindstedt,1956)

and are therefore probably intermediates in the breakdown of cholesterol to bile acids. The work present here further strengthens this evidence.

The reduction of cholest-4-en-3-one-7α-ol is found to take place in a purified preparation of the supernatant fraction of rat liver. This preparation, used by Tomkins (1956, 1957) has been shown to be non-specific, in that it will readily reduce C_{21} steroids such as progesterone and cortisone, but it shows little or no activity in reducing cholest-4-en-3-one. On further purification of this fraction Tomkins found that, contrary to his expectation of finding one non-specific reductase several specific reductases do exist in the supernatant fraction. Possibly on further purification of the "supernatant Enzyme" a specific reductase for cholest-4-en-3-one-7α-ol might be found.

Tomkins (1956, 1957) found that this purified supernatant fraction (the protein precipitating between 50% and 70% saturation with ammonium sulphate) converted cortisone firstly to dihydiocortisone and then to tetrahydrocortisone (see diagram 6). Thus it would be expected that

cholest-4-en-3-one-7α-ol would be converted to coprostan-3-one-7α-ol and then further to 3α,7α-dihydroxycoprostane. Danielsson (1961 c) has in fact suggested that coprostan-3-one-7α-ol is an intermediate in cholesterol metabolism as it is converted to both chenodeoxycholic acid and cholic acid in the bile-fistula rat.

However, on incubation of cholest-4-en-3-on-7α-ol with this fraction two products were isolated, one of which was 3α,7α-dihydroxycoprostane and the other was cholest-4-en-3α,7α-diol. Initially on the basis of this evidence it was thought that cholest-4-en-3-one-7α-ol was reduced at the 3-keto group first, leaving the double-bond intact, and then dihydroxycoprostane was produced by reduction of the double-bond. However, studies on the incubation of cholest-4-en-3α,7α-diol with this fraction did not seem to support this theory, as it was found to be converted only in very small amounts to dihydroxy-coprostane. Also inhibitor studies with cholest-4-en-3-one, showed that dihydroxycoprostane production was inhibited whereas no effect could be seen on the production of cholest-4-en-3α,7α-diol.

The work carried out using coprostan-3-one-7α-ol as substrate for this supernatant enzyme, however, clarified the situation. This substance was found to be converted very readily to 3α,7α-dihydroxycoprostane and therefore it seems that two series of reactions occur when cholest-4-en-3-one-7α-ol is incubated with this fraction. Firstly, cholest-4-en-3-one-7α-ol is converted to coprostan-3-one-7α-ol with NADPH as co-factor (NADH cannot replace NADPH as the production of dihydroxycoprostane does not occur when NADH is used). As coprostan-3-one-7α-ol is converted so readily to dihydroxycoprostane, it does not accumulate and cannot therefore be isolated from an incubation. The reduction of the 3-keto group of coprostan-3-one-7α-ol requires either NADPH or NADH as co-factor. Secondly, cholest-4-en-3-one-7α-ol can be reduced at the 3-keto group first to yield cholest-4-en-3α,7α-diol, the reaction being specific for NADPH as co-factor. This substance, as it is not converted easily to 3α,7α-dihydroxy-coprostane, accumulates in an incubation and can be isolated. Cholest-4-en-3-one, presumably inhibits only the enzyme involved in the reduction of cholest-4-en-3-one-7α-ol to coprostan-3-one-7α-ol.

Reduction of the 3-keto group to a 3α-hydroxyl group, before reduction of the double-bond, does not apparently occur when C_{21} steroids, such as cortisone are used as substrates. However, Ringold, Ramachandran and Forchielli (1962) have noted that at pH 5.8, rat liver supernatant, in the presence of NADPH can convert certain halogenated Δ^4-3-keto steroids (2α-fluoro, 6β-fluoro, 6α-fluoro and 4-chloro) to the corresponding allyl alcohols, the 3α-epimer being the predominant product. Testosterone and androsterone, however, were not reduced in this way. Also cholest-4-en-3α,7α-diol has never been suggested to be an intermediate in cholesterol breakdown to bile acids. However, this substance could be an intermediate in the formation of bile acids containing a hydrogen atom in the 5α-position (i.e. allo-bile acids) to which more importance has been given recently, as new methods for their separation from the "normal" bile acids (5β) have been developed. It would be of interest therefore to study the metabolism of this substance both <u>in vitro</u> and <u>in vivo</u> in the bile fistula rat.

Preliminary experiments have shown that 3α,7α-dihydroxycoprostane is converted to a more polar product in the mitochondrial fraction of rat liver. This product is thought to be the 26-hydroxylated derivative and not 3α,7α,12α-trihydroxycoprostane which was slightly more polar than the incubation product. This is in accord with the studies of Berseus and Danielsson (1963) who found that dihydroxycoprostane was 26-hydroxylated in mouse mitochondria and that the product, coprostan-3α,7α,26-triol was converted to chenodeoxycholic acid in the bile-fistula rat.

12α-hydroxylation would seem to be a very difficult reaction to reproduce in an in vitro system and no 12α-hydroxy derivatives have been reported to be produced in vitro studies. However, a more detailed study of the metabolism of dihydroxycoprostane and the effect of SF, might yield information on this important reaction. Dihydroxycoprostane, as it can be converted to either chenodeoxycholic acid or cholic acid (Bergstrom and Lindstedt, 1956) then, may lie at the division in the pathway, one branch of which will end in chenodeoxycholic and the other branch in cholic acid.

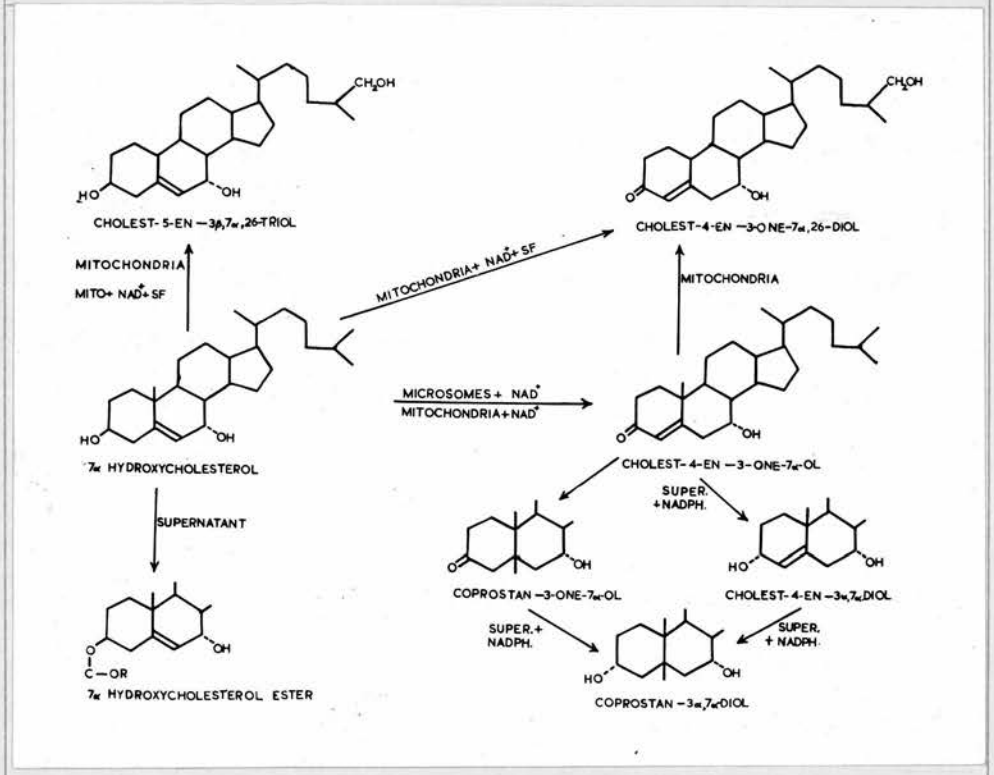

CHOLEST-5-EN-3β,7α,26-TRIOL
MITOCHONDRIA
MITO+ NAD+ SF
MITOCHONDRIA+ NAD+ SF
CHOLEST-4-EN-3-ONE-7α,26-DIOL
MITOCHONDRIA
7α HYDROXYCHOLESTEROL
MICROSOMES+ NAD+
MITOCHONDRIA+NAD+
CHOLEST-4-EN-3-ONE-7α-OL
SUPERNATANT
SUPER. +NADPH.
COPROSTAN-3-ONE-7α-OL
CHOLEST-4-EN-3α,7α-DIOL
SUPER.+ NADPH.
SUPER. + NADPH.
7α HYDROXYCHOLESTEROL ESTER
COPROSTAN-3α,7α-DIOL

Fig. 52

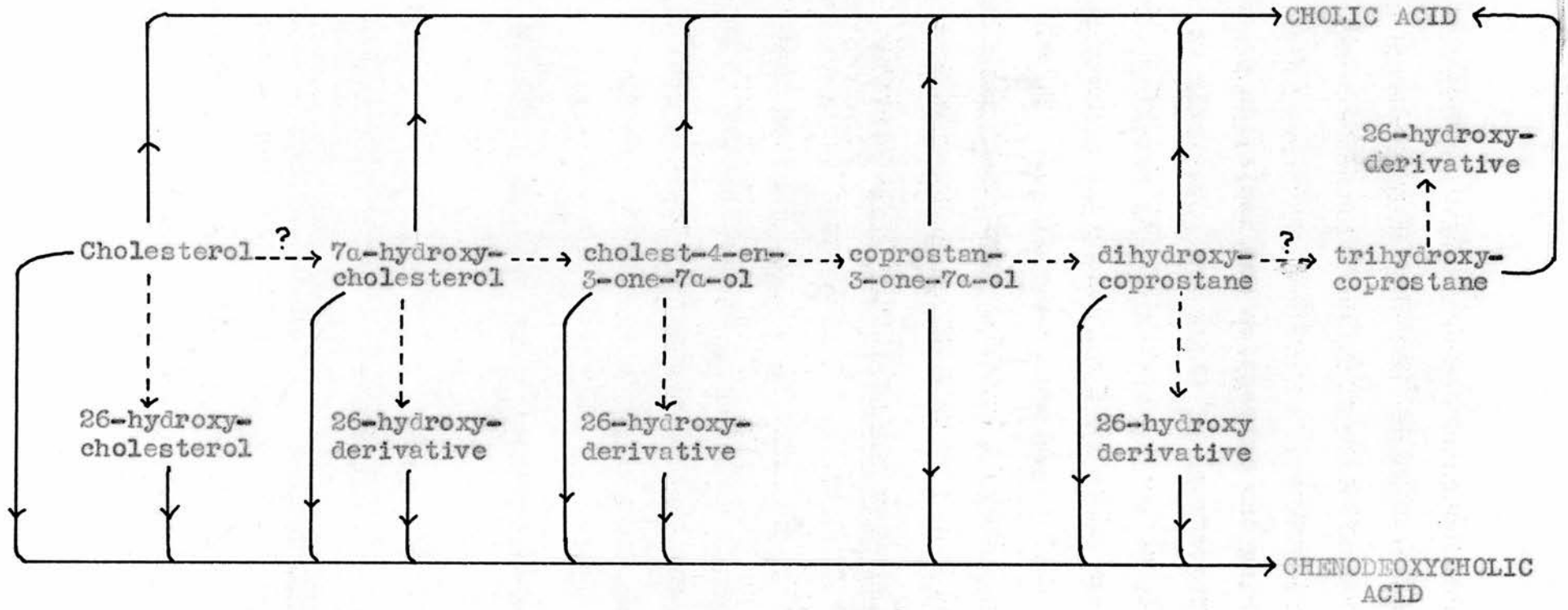
Fig. 52 summarises the reactions which have been shown to occur as a results of this work and the cell fractions in which they proceed.

The sequence of events in the breakdown of cholesterol could be visualised as taking place in an orderly fashion without the steroid having to be transported from one part of the cell to another. It is not knownwhere in the cell 7α-hydroxylation of cholesterol occurs, but it has been shown here that the most efficient conversion of 7α-hydroxycholesterol to cholest-4-en-3-one-7α-ol takes place in the microsomal fraction. Hydroxylation at position 26 of either 7α-hydroxy-cholesterol or cholest-4-en-3-one-7α-ol takes place in the mitochondria, but in the latter case the activity has been shown to lie in the mito-chondrial debris. Thus it is possible for the steroid to be positioned in the cell sap so that hydroxylations (at position 26) can take place at one end of the molecule by enzymes in the mitochondrial wall whereas dehydrogenation and isomerisation could take place to form cholest-4-en-3-one-7α-ol at the other end of the molecule by enzymes in the microsomes. Hydroxylation at the 26-position has also been shown to be

catalysed by microsomal enzymes on the addition of a co-factor from the supernatant. This hydroxylating system may be more important than that located in the mitochondria in which case all the reactions discussed can occur if the steroid is positioned in the supernatant (or cell sap) and alongside the endoplasmic-reticulum. Reduction of cholest-4-en-3-one-7α-ol could then also take place as the enzymes catalysing these reactions are found in the supernatant. The cleavage of the side-chain of cholesterol to give CO_2 takes place in the mitochondria, together with a co-factor (SF) from the supernatant (Whitehouse et al. 1961). This reaction is not affected by sonication of the mitochondria and thus may also occur in the mitochondrial wall.

In liver there exists a large cholesterol pool, about 15 mg. in a 200 g. rat. Quantitatively, the most important route of breakdown of cholesterol is to the bile acids in liver, about 5 mg. being formed per day in a 200 g. rat. The steps in this breakdown presumably occur in a smooth and organised fashion as no intermediates on the pathway have ever been found to accumulate in rat liver, except 7α-hydroxycholesterol which

Fig. 53



---- Reactions shown to occur, in in vitro studies

—— Reactions shown to occur from studies on bile-fistula rats.

has been isolated together with the other auto-oxidation products, 7-ketocholesterol, 7β-hydroxycholesterol and their dehydration products. These autoxidation products have probably been formed during the extraction and isolation procedure. Therefore, if there are apparently no slow reactions in the pathway it is puzzling to find in this study that the reactions proceed in different cell fractions of rat liver. In the preceeding paragraph an attempt has been made to explain this, but it must be emphasised that *in vitro* studies are difficult to interpret. *In vivo* studies on bile-fistula rats designed to identify intermediates have also provided useful information on cholesterol metabolism but it must be remembered that in these bile-fistula rats cholesterol breackdown is abnormally rapid. However, taking the results of *in vivo* and *in vitro* studies together, a picture of the steps involved in cholesterol breakdown can be built up (Fig. 53).

From the *in vitro* work done in this study, it seems that oxidation and reduction reactions proceed very efficiently on the addition of the appropriate co-factors, whereas hydroxylation does not proceed so easily. This could be interpreted

in several ways. For instance, it could mean that the affinities of the oxidation and reduction enzymes for the appropriate substrates are greater than those of the hydroxylation enzymes. Alternatively oxidation and reduction may be less complicated reactions than hydroxylation and therefore would be more easily reproduced *in vitro*. Again, the hydroxylating system, *in vivo* might be more efficient than oxidation and reduction, but being a complicated system (for which there is evidence) the procedure used in *in vitro* studies would very likely inactivate part of the system so that hydroxylation appears to be an inefficient reaction.

None of the enzymes involved in the breakdown of cholesterol have ever been purified and characterised, although the reductase system in the supernatant has been partially purified in this study. This means that, firstly no mechanism for hydroxylation, for example, can be suggested in such a crude system, although it can be said that 26-hydroxylation takes place in mitochondria or microsomes and that a co-factor found in the supernatant fraction is involved. Secondly the specificity of an enzyme system towards

several substrates is difficult to test. Thirdly any reaction studied is complicated by other reactions occurring simultaneously and possibly interfering. This is illustrated by the situation in mitochondrial incubations where the NAD level is so important in deciding whether oxidation or hydroxylation of 7α-hydroxycholesterol will occur. It is impossible to tell what the level of NAD will be in the living rat and therefore it is not knownwhich of the two reactions, oxidation or hydroxylation will normally be occurring.

One hormonal effect on cholesterol breakdown has been studied and this is the influence of thyroxine. Eriksson (1957) in a study on the biliary excretion of both cholic and chenodeoxycholic acid in eu-, hyper- and hypothryoid bile fistula rats, found a decreased secretion of cholate in both hypo- and hyperthyroid rats. Also a substantial increase in the amount of chenodeoxycholate formed in hyperthyroid rats was seen and the chenodeoxycholate excreted in hypothryoid rats was less than that in euthyroid rats. These results point to the possibility of

a direct inhibitory effect of thyroxine on the 12α-hydroxylase or of a stimulatory effect on side-chain oxidation. However, no hormonal effects on any single reaction in cholesterol breakdown, have been studied *in vitro*. As 12α-hydroxylation has not been demonstrated *in vitro* it may be a very complicated reaction, perhaps influenced by many co-factors, among which might be thyroxine.

Cholesterol, 7α-hydroxycholesterol, cholest-4-en-3-one-7α-ol and dihydroxycoprostane have all been shown to be hydroxylated in the 26-position, the 26-hydroxylated derivatives then forming only chenodeoxycholic acid in the bile-fistula rat. It is therefore impossible to say whether the 26-hydroxylase system is very non-specific or whether a separate enzyme does exist for each of these substrates. If the former instance is the case, then the substrate which *in vivo* is 26--hydroxylated is unknown and will remain unknown until the enzyme system is purified. On the other hand if each substrate is 26-hydroxylated *in vivo* a series of oxidation and reduction enzymes must exist which will transform the 3β-hydroxyl group to a 3α-hydroxyl group with the 26-hydroxylated

derivatives as substrates. It may be, of course, that the oxidation and reduction enzymes studied in this work catalyse the oxidation and reduction of the 26-hydroxy derivatives more efficiently than the compounds studied. Again this possibility cannot be tested until purified enzyme preparations are obtained. Also, for the same resons, it is impossible to say which substrate is 12α-hydroxylated *in vivo*.

This work has shown that in the breakdown of cholesterol to bile acids certain definite variations in metabolic routes exist. Further studies employing purified cell fractions and better methods of sterol assay may throw light on the relative quantitative importance of these routes. The involvement of the co-enzymes, NAD and NADP, in the various stages of breakdown of cholesterol, suggests the possibility of control of the various pathways by the transhydrogenase system which in turn may possibly be a site of action of certain hormones. This work illustrates that the mechanism of hydroxylation is complex and perhaps many enzymes and co-factors may take part in this process. A new co-factor involved in hydroxylation reactions remains to be identified.

Some suggestions for further work:

a) As growing rat macrophages have been shown to metabolise cholesterol or its esters, it would be advantageous to study the metabolism of 7α-hydroxycholesterol and other intermediates in bile acid formation in this type of tissue preparation.

b) As cholesterol is so prone to autoxidation and the study of its metabolism <u>in vitro</u> therefore made difficult more information on cholesterol breakdown might be gained by carrying out a study on the metabolism of cholesterol esters, which are not readily autoxidised. Also it is possible that cholesterol has to be esterified before breakdown can occur.

c) An attempt to identify the co-factor SF, which seems to play a part in hydroxylation reactions would be of great interest, and might lead to an insight into the mechanism of hydroxylation. However, a hydroxylating system which could be studied by a fast assay procedure is required before much progress can be made in identifying SF.

d) A study of the effect of anaerobic conditions on hydroxylation reactions and the effect of SF under such conditions might yield information on the nature of SF, and indicate whether it might be a bound form of oxygen.

e) The chemical syntheses of 12-hydroxylated derivatives of cholesterol could be undertaken and their breakdown or formation studied in the cell fractions of rat liver, it would be of interest to discover whether SF has a role also in 12α-hydroxylation.

f) A detailed study of the esterification of 7α-hydroxycholesterol and the significance of this reaction might yield considerable information.

SECTION VIII

APPENDICES

APPENDIX I

CHEMICAL SYNTHESES

1. 7 α-hydroxycholesterol.

This substance was synthesised from cholesterol acetate, which was reacted with N-bromosuccinimide to form the 7-bromo-compound. This was converted to 7 α-hydroxycholesterol by the method of Henbest and Jones (1948) using sodium formate and formic acid. A mixture of 7 α- and 7 β-hydroxycholesterol was obtained on hydrolysis and the isomers were separated by column chromatography on silicic acid. The mixture was added to the column in 70 % chloroform : 30% petrol ether and the column was eluted with increasing amounts of chloroform in petrol ether and 7 α-hydroxycholesterol was eluted with 100% chloroform. The purified substance ran as one spot in several thin layer chromatography systems (see page 312), spraying blue with phosphotungstic acid.

Melting point = 183 - 184°C.

[Published melting point = 184°C.,
Barr, Heilbron, Parry and Spring (1936)]

50 mg of the pure 7 α-hydroxycholesterol was then labelled with tritium using the Wilzbach method (Wilzbach, 1957). This method depends on the exchange

of tritium atoms with hydrogen atoms in the substance to be labelled. The apparatus consists of a conical flask containing the steroid, spread over as large a surface area as possible in the bottom of the flask, and connected by glass tubing to an unbroken tritium ampoule. The whole system is arranged so that it can be evacuated. After evacuation, the ampoule of tritium gas was broken from the outside by using a ball-bearing inside and a magnet outside. The apparatus was left under vacuum for about three days, during which the tritium exchanged with the hydrogen atoms of the steroid molecules. The steroid was removed from the apparatus, the labile tritium removed, and,as many polar degradation products were formed during the labelling procedure, the 7 α-hydroxycholesterol was re-purified by column chromatography on silicic acid. The purity of the compound was checked by running on a thin layer plate, segmenting the plate and counting each segment. The radioactivity was found to be associated only with the 7 α-hydroxycholesterol. The specific activity of the pure compound was found to be 500,000 c/m/mg. (Melting point = 183° C.)

2. Cholest-4-en-3-one-7 α-ol.

The substance was synthesised by Mr. Naqui. Cholesterol was oxidised with benzoquinone to yield cholest-4:6-dien-3-one, which was further converted to cholest-4-en-6 α,7 α -epoxide by treatment with monoperphthalic acid. The epoxide was reduced with lithium aluminium hydride producing cholest-4-en-3α,7α-diol, which, on oxidation with manganese dioxide, gave cholest-4-en-3-one-7α -ol.

Melting point = 183 -184°C. [Published melting point = 183 - 184°C. Danielsson (1961 b)]

Optical rotation = -67°

Wavelength of maximum absorption = 242 mμ ,

ϵ_{242} = 15,500

Carbon	80.92%	Theory	80.1%
Hydrogen	11.20%	Theory	11.0%

This substance was also tritiated by the Wilzbach method, and re-purified by column chromatography. Its purity was checked by running a thin layer plate in the same way as for 7 α-hydroxycholesterol. The specific activity of the pure compound was found to be 500,000 c/m/mg.

It was found that leaving substances to exchange with tritium for periods of more than a week led to the production of so many degradation products that it was

quite impossible to purify the original steroid. Thus, substrates were left for only about three days, when they were found to have a sufficiently high specific activity, and could be readily purified.

3. Cholest-5-en-3 β-ol-7 α-hydroperoxide. (Prepared by Mr.Naqui)

Cholesterol was subjected to photo-oxygenation in pyridine solution with haematoporphyrin as catalyst (Schenk, Gollnick and Neumüller, 1957), to yield cholest-6-en-3 β-ol-5 α -hydroperoxide. This substance was rearranged in chloroform solution to give cholest-5-en-3 β -ol-7 α -hydroperoxide.

Melting point = 147°C.

Optical rotation = -137°.

Carbon : 77.25% Theory : 77.45%

Hydrogen : 10.76% Theory : 11.08%

4. Cholest-4-en-3 α ,7 α -diol. (Prepared by Mr. Naqui)

Cholesterol was oxidised with benzoquinone to yield cholest-4:6-dien-3-one, which was further converted to cholest-4-en-6 α,7 α -epoxide by treatment with monoperphthalic acid. On reduction with lithium aluminium hydride, cholest-4-en-3 α,7 α -diol was produced.

Melting point = 98°C. Optical rotation = -135°

Carbon : 79.64% Theory : 80.53%

Hydrogen : 11.51% Theory : 11.44%

5. Coprostan-3α,7α-diol. (3α,7α-dihydroxycoprostane) (Prepared by Mr.Naqui)

(a) Cholesterol was converted to cholest-4-en-6α,7α-epoxide as above, which was then reduced at room temperature with hydrogen gas and 10% palladium on charcoal as catalyst to yield coprostan-3-one-7α-ol. This was further reduced with lithium aluminium hydride to yield both coprostan-3α,7α-diol and the corresponding 3β-epimer. The 3β-epimer was removed by precipitation with digitonin, leaving coprostan-3α,7α-diol.

Melting point = 84 - 86°C. (Published melting point = 84 - 86°C., Bergstrom and Krabisch, 1957).

(b) Coprostan-3α,7α-diol was also prepared by the author by electrolytic condensation of chenodeoxycholic acid with isovaleric acid.

Melting point = 80 - 82°C.

6. Coprostan-3-one-7α-ol. (Prepared by Mr.Naqui)

This was prepared by two methods; firstly as described in 5(a), and secondly by reduction of cholest-4-en-3-one-7α-ol with hydrogen and platinum at -27°C.

Melting point = 121°C. (Published melting point = 121°C., Yamasaki, Kawahara and Shimizu, 1959)

Carbon : 80.84% Theory : 80.67%

Hydrogen : 11.37% Theory : 11.44%

7. Cholest-5-en-3 β,7∝,26-triol. (Prepared by Mr.Naqui)

This was prepared from the plant sterol kryptogenin, which, after a Clemmenson reduction, yielded two products, cholest-5-en-3 β,26-diol (26-hydroxycholesterol), and cholest-5-en-16-one-3 β,26-diol. The mixture was reduced giving only 26-hydroxycholesterol, which was then subjected to a photo-oxygenation reaction in pyridine with haematoporphyrin as catalyst, giving cholest-5-en-3 β,26-diol-7∝-hydroperoxide. This was reduced with lithium aluminium hydride giving cholest-5-en-3 β,7∝,26-triol.

APPENDIX II

CHARACTERISTICS OF STEROID CHROMATOGRAPHIC STANDARDS

Steroid	Melting Point °C.		Max. Absorption mμ	Extinction coeff.	Source
	Obs.	Pub.			
1. Cholesterol	149	149			Dr.G.S.Boyd
2. Cholest-4-en-3-one	81	82	240	18,000	Dr.G.S.Boyd
3. Trihydroxycoprostane	184	185 -186			Dr.G.S.Boyd
4. 26-Hydroxycholesterol	176	177 -179			Mr.M.Naqui
5. Cholest-4:6-dien-3-one	81	82	284	27,000	Mr.M.Naqui
6. 7-Ketocholesterol	170	170	238	14,000	Dr.G.S.Boyd

References:

2. Fieser (1955)

3. Kazuno and Mori (1954)

4. Scheer, Thompson and Mosettig (1956)

5. Mandell (1956)

6. Fieser, Fieser and Chakravarti (1949)

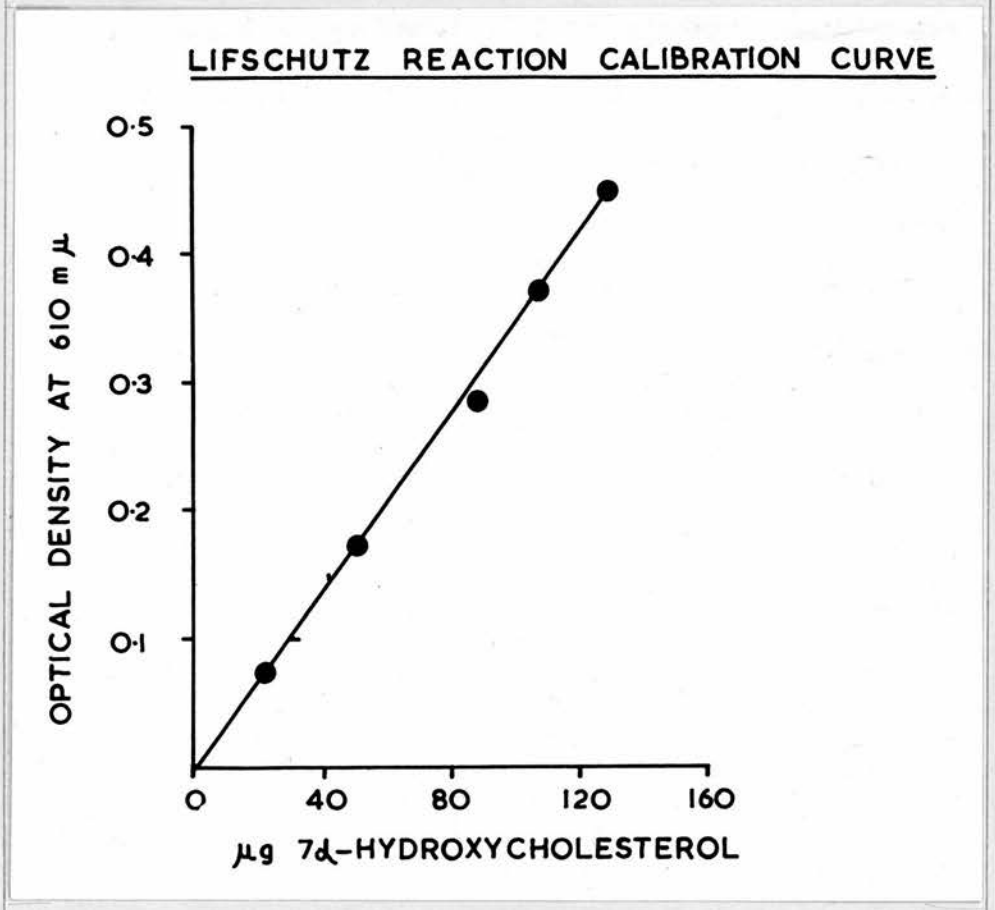

LIFSCHUTZ REACTION CALIBRATION CURVE
OPTICAL DENSITY AT 610 mμ
0·5
0·4
0·3
0·2
0·1
0
40
80
120
160
μg 7α-HYDROXYCHOLESTEROL

Fig. 51

APPENDIX III

LIFSCHUTZ COLOUR REACTION

The reagent consists of a solution of 1 g ferric chloride in 900 ml glacial acetic acid and 100 ml concentrated sulphuric acid, as described by Bergstrom and Wintersteiner (1942). With 7α-hydroxycholesterol and some other substances (see Table 39), a blue colour with a maximum absorption between 610 and 640 mμ is obtained. A solution of the substance to be estimated is dissolved in 1.5 ml dry chloroform and 3 ml of the reagent are added. The blue chromogen is read at 610 mμ after 10 minutes against a reagent blank. The relation between light absorption and concentration is linear from 20 μg to 150 μg for 7 α-hydroxycholesterol, although at greater concentrations relatively more colour is produced (see calibration curve, figure 51). Thus, estimations of material are always calculated to come within the above range.

Bergstrom and Wintersteiner (1942) suggested that a 2:4:6-triene or cholest-4:6-dien-3-ol may be the ultimate precursor of the blue pigment. Allylic shifts, such as from a cholest-5-en-3:7-diol to a cholest-6-en-3:5-diol and subsequent dehydration could easily occur in strong acid.

TABLE 39

REACTION OF SOME STEROIDS WITH LIFSCHÜTZ REAGENT AND WITH PHOSPHOTUNGSTIC ACID

Compound	Reaction with Lifschutz Reagent	Colour with phospho-tungstic acid
Cholesterol	-	pink
Cholesterol esters	-	pink
Cholest-4-en-3-one	-	-
Cholest-5-en-3-one	-	pink
Cholestan-3-one	-	-
Coprostan-3-one	-	-
Cholest-5-en-7-one	-	-
Cholestan-3β-ol	-	pink on prolonged heating
Coprostan-3β-ol	-	pink on prolonged heating
Cholestan-3α-ol	-	pink on prolonged heating
Coprostan-3α-ol	-	pink on prolonged heating
Cholest-5-en-3β-7α-diol	+	blue, very readily
Cholest-5-en-3β,7α-diol esters	+	blue, very readily
Cholest-5-en-3β,7β-diol	+	blue, readily
Cholest-4-en-3-one-7α-ol	-	pink

Table continued on next page.

TABLE 39 (cont.)

Compound	Reaction with Lifschutz Reagent	Colour with phospho-tungstic acid
Cholest-4-en-3 α,7α-diol	+	blue
Cholest-4-en-3 β,7α-diol	+	blue
Cholest-5:7-dien-3 β -ol	+	bluish-purple
Cholest-3:5-dien-7-one	-	pink
Cholest-4:6-dien-3-one	-	-
Coprostan-3 α,7α-diol	-	yellow, on prolonged heating
Coprostan-3 α,7α ,12 α-triol	-	yellow, on prolonged heating
Coprostan-3-one-7α-ol	-	-
Cholest-5-en-3 β,26-diol	-	pink
Cholest-5-en-3 β-ol-7α-hydroperoxide	-	purple
Vitamin A	-	-
Vitamin A palmitate	+ (transient)	bluish-grey

APPENDIX IV

THIN LAYER CHROMATOGRAPHY

R_F VALUES OF SUBSTANCES IN DIFFERENT SOLVENT SYSTEMS

Substance	R_F values Solvent system			Method of detection
	Benzene (2) Ethyl acetate(1)	Benzene(10) Ethyl acetate(5) Acetone(3)	Benzene (19) Dioxane (1)	
Cholesterol	0.62	0.85	0.36	Pink p't
Cholesterol esters	1.0	1.0	1.0	Pink p't
7 α-Hydroxycholesterol	0.14	0.35	0.05	Blue p't
7 β-Hydroxycholesterol	0.26	0.42	0.05	Blue p't
7 α-Hydroxycholesterol esters				
- acetate	1.0	1.0	0.24	Blue p't
- laurate	1.0	1.0	0.38	Blue p't
- stearate	1.0	1.0	0.63	Blue p't
Cholest-4-en-3-one-7 α-ol	0.42	0.66	0.08	u.v.;pink p't
Cholest-4-en-3-one	0.80	0.89	0.50	u.v.,blue p'm
Cholest-5-en-3 β,26-diol	0.36	0.55	0.073	Pink p't
Cholest-5-en-3 β-ol-7-one	0.36	0.56	0.08	u.v.;blue p'm
Coprostane-3-one-7 α-ol	0.65	0.85	0.33	Blue p'm
Cholest-4-en-3 α,7 α-diol	0.15	0.35	0.05	Blue p't
Coprostan-3 α,7 α-diol	0.20	0.43	0.06	Yellow p't
Coprostan-3 α,7 α,12 α-triol	0	0.14	0	Yellow p't
Cholest-4:6-dien-3-one	0.8	0.89	0.53	u.v.;pink p't
Cholest-5-en-3 β-ol-7 α-hydroperoxide	0.36	0.58	0.09	Purple p't
Cholest-5-en-3 β,7 α,26-triol	0	0.15	0	Blue p't

In the above Table

p't = phosphotungstic acid; p'm = phosphomolybdic acid;

u.v. = absorbs when viewed in u.v. light.

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Detection of steroids on chromatoplates using a non-destructive method

The excellent resolution and rapid development times of thin-layer chromatography make this technique a very useful tool in the steroid field. As many sterols and steroids have characteristic absorption bands in the ultraviolet *e.g.* cortisone, progesterone, cholestenone etc., advantage may be taken of this fact to develop a method whereby these substances can be detected, and recovered quantitatively from a thin-layer plate without the use of a destructive spraying reagent. This is of importance in steroid studies *in vitro* where the material has to be quantitatively eluted, chemically determined and also assayed for radioactivity.

Thin-layer chromatography was carried out in the usual way except that quartz plates were used instead of glass plates. The plates were viewed with a mercury lamp (maximum emission 254 mμ) and ultraviolet-absorbing substances appeared as dark spots. However, the silicic acid layer itself absorbed (or scattered) the incident light and so the contrast between spots and background was not very great. This meant that the method had a low order of sensitivity.

SEASE[1] incorporated an inorganic phosphor into silicic acid and used the mixture in a quartz column. By viewing the column under ultraviolet light he observed the presence of ultraviolet-absorbing bands. KIRCHNER[2] adapted this method to thin-layer chromatography of terpenes using glass plates for the chromatography.

This method was modified to permit its use in the detection of steroids as follows. Various inorganic phosphors which fluoresce green under the mercury ultraviolet lamp were mixed with Silica Gel G (Merck) and used for thin-layer chromatography of steroids on glass plates. The silicic acid layer was exposed to an ultraviolet source and the chromatoplate examined from the "glass side". Absorbent substances having maximum absorption between 240 and 280 mμ show up as dark spots which can be marked on the glass side, eluted and either estimated spectrophotometrically and/or assayed for radioactivity. Since the wavelength of the emission from the phosphor varies with the wavelength of the ultraviolet light used to illuminate the plates the background colour changes with some phosphors from bluish green at 200 mμ to green at 240 mμ and to red at 300 mμ.

This method is particularly applicable to the study of intermediary metabolism of adrenal steroids and oestrogens and also to the study of the breakdown of cholesterol to bile acids where several of the expected intermediates are ultraviolet absorbing. For example, using this thin-layer technique coupled to a preliminary column method it can be shown that incubation of 7α-hydroxycholesterol with certain cell fractions of rat liver gives rise to cholest-4-en-3-one-7α-ol (*cf.* YAMASAKI[3]; DANIELSSON[4]) with maximum absorption at 242 mμ, while incubation of cholest-4-en-3-one-7α-ol produces a more polar ultraviolet-absorbing substance (maximum absorption at 240 mμ) which is possibly cholest-4-en-3-one-7α-26(?)-diol. Using this method, incubations can be carried out with very small amounts, in some cases less than 25 μg of substrate either unlabelled or labelled with tritium and the products of the reaction detected and quantitatively estimated. These substrates and products cannot be separated as quickly or in such small amounts using conventional column methods.

The method can be made more specific by varying the wavelength of the ultraviolet light used to illuminate the plate. High-intensity ultraviolet sources and gratings

are required for this purpose because the energy of the monochromatic light emerging must be considerable to illuminate effectively even small plates.

Using a hydrogen lamp, substances which maximally absorption at 207 mμ such as cholesterol and 7α-hydroxycholesterol, can easily be detected. Thus by using a series of interference filters in the range 200–300 mμ with a hydrogen source, this method not only shows the position of a substance on the plate but indicates its ultraviolet-absorbing properties and thus gives information on its possible molecular structure.

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